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No. 10-426

IN THE

Supreme Court of the United States

APPLERA CORP. AND TROPIX, INC.,
Petitioners,

v.

ENZO BIOCHEM, INC., ENZO LIFE SCIENCES, INC., AND YALE UNIVERSITY,
Respondents.

On Petition for a Writ of Certiorari to the United States Court of Appeals for the Federal Circuit

BRIEF IN OPPOSITION

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QUESTION PRESENTED

Whether this Court should overrule *Eibel Process Co.* v. *Minnesota & Ontario Paper Co.*, 261 U.S. 45 (1923), and hold that 35 U.S.C. § 112's requirement of "claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention" precludes the use of relative terms like "substantially" and "approximately" in claims even though a person skilled in the art would understand, in light of the entire patent and existing knowledge, what is being claimed.

CORPORATE DISCLOSURE STATEMENT

Pursuant to Supreme Court Rule 29.6, respondents state at follows:

Enzo Biochem, Inc. is the parent of Enzo Life Sciences, Inc. Otherwise, there are no parents or corporations holding 10% or more of the stock of any of respondents.

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BRIEF IN OPPOSITION

PRELIMINARY STATEMENT

The petition in this case is premised on the mistaken notion that the "claims" of a patent issued by the U.S. Patent and Trademark Office ("PTO") should operate as blueprints for circumventing the rights of inventors. But "the certainty which the law requires in patents is not greater than is reasonable." *Minerals Separation* v. *Hyde*, 242 U.S. 261, 270 (1916). Thus, "[e]xpressions quite as indefinite as 'high' and 'substantial,' in describing an invention or discovery, in patent specifications and claims, have been recognized by this court as sufficient."

Eibel Process Co. v. *Minn. & Ontario Paper Co.*, 261 U.S. 45, 66 (1923). Indeed, this Court has *never* invalidated patent claims simply because they are amenable to two reasonable constructions. To the contrary, "if the claim were fairly susceptible of two constructions, that should be adopted which will secure to the patentee his actual invention." *Smith* v. *Snow*, 294 U.S. 1, 14 (1935). Consistent with (and in reliance on) those holdings, inventors have for decades sought and obtained patents with claim terms like "substantially," "approximately," and "about." In view of this Court's precedents and decades of reliance on those precedents, *stare decisis* answers the question presented. See *Hilton* v. *S.C. Pub. Rys. Comm'n*, 502 U.S. 197, 206-207 (1991); *Payne* v. *Tennessee*, 501 U.S. 808, 828 (1991). That alone is sufficient reason to deny the petition.

Largely ignoring this Court's precedents, petitioners urge that the standard of "definiteness" allegedly employed by the decision below conflicts with the statutory requirement of distinct and particular claims. But the standard they assail—which suggests a claim is indefinite if it is "insolubly ambiguous"—was invoked nowhere by the decision below. Rather, that decision held that the claims were not indefinite because "those skilled in the art would understand what is claimed," Pet. App. 11a, a standard petitioners nowhere challenge.

Besides, petitioners misread the Patent Act. The language petitioners invoke was inserted to require applicants to clearly distinguish their inventions not from the universe of potentially infringing activities but rather from *other* subject matter disclosed in the patent that is *not* being claimed as the novel and useful invention. See *Merrill* v. *Yeomans*, 94 U.S. 568, 570 (1876). Thus, an inventor must state what the invention "is": Where the invention is part of a machine or composition of matter that combines the old with the new, the inventor must

identify what parts of the machine or composition constitute the claimed invention. But petitioners' proposal that claims must mark unmistakable boundaries for what the invention is "not" lacks foundation. Indeed, it is impossible to reconcile with the "doctrine of equivalents," which gives patentholders the right to exclude others from practicing not only what is expressly defined by the claims but also "all equivalents." That doctrine "renders the scope of patents less certain" but is needed to preserve the value of the patent. *Festo Corp.* v. *Shoketsu Kinzoku Kogy Kabushiki Co.*, 535 U.S. 722, 732 (2002). In any event, the patent in this case distinctly points out the invention being claimed; the purported ambiguity petitioners invoke is not one that would lead to reasonable confusion.

The assertion of importance likewise fails. For more than a decade, the Federal Circuit has addressed indefiniteness under settled standards without criticism by its active judges. While the standard differs from the one the PTO applies to applications, patent examination and patent enforcement are different. The PTO has explained that its rules are designed to force applicants to amend and improve their claims during an interactive patent examination process. Courts, by contrast, must either accept or invalidate patents the PTO has already approved. Regardless, both the PTO and Federal Circuit agree that the disputed claim language here satisfies statutory requirements.

Finally, this case is an entirely unsuitable vehicle for addressing the question presented. The decision's interlocutory posture "alone furnishe[s] sufficient ground for the denial" of the petition. *Hamilton-Brown Shoe Co.* v. *Wolf Bros. & Co.*, 240 U.S. 251, 258 (1916); see *Va. Military Inst.* v. *United States*, 508 U.S. 946 (1993) (Scalia, J., concurring). And this case does not present a genuine issue of indefiniteness. Petitioners object to a

single segment of a novel class of molecules that are otherwise claimed with definite structural specificity. That disputed piece, a "linkage group" that does not "substantially interfere" with hybridization, defines a boundary the public would never seek to approach, because hybridization is *necessary* to the function of those molecules. The indefiniteness challenge is thus based on the phony premise that petitioners want to figure out, with certainty, how to avoid the patent by building a molecule that, because it has obstructed hybridization, is effectively broken from the outset.

STATEMENT

I. STATUTORY BACKGROUND

The Patent Acts of 1790 and 1793 required inventors to "distinguish" their inventions from "other things before known." Act of Apr. 10, 1790, ch. 7, §2, 1 Stat. 109; Act of Feb. 21, 1793, ch. 11 §3, 1 Stat. 318. Neither expressly required inventors to distinguish those inventions using "claims." See *Markman* v. *Westview Instruments, Inc.*, 517 U.S. 370, 379 (1996).[1] In this country, the practice of using "claims" to assist in identifying the invention derived from *Evans* v. *Eaton*, 20 U.S. 356 (1822). See 3 *Chisum on Patents* §8.02[1] (2009). In that case, the Court addressed whether subject matter must be identified as inventive or whether novelty "may be established by comparing the machine specified in the patent with former machines in use." 20 U.S. at 433. Construing the 1793 Act's requirement that inventors "distinguish" their inventions from prior art, the Court held that the Act did not authorize a patent "[w]hich

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[1] Patent practitioners typically refer to the numbered paragraphs, set forth at the end of U.S. Patents, as the "claims." The written description of the invention that appears before the separately numbered claims, together with the claims themselves, is referred to as the "specification."

describes the machine fully and accurately, as a whole, mixing up the new and old, but does not *in the slightest degree* explain what is the nature or limit of the improvement which the party claims as his own." *Id.* at 434 (emphasis added).

From that decision, "claim" practice—the practice of identifying the part of a machine, process, etc., that is claimed as novel and patentable in numbered "claims"—has developed. The practice received statutory recognition in the Patent Act of July 4, 1836, ch. 357, § 6, 5 Stat. 119, see *Markman*, 517 U.S. at 379, which required an inventor to "particularly specify and point out the part, improvement, or combination which he claims as his own invention or discovery." Such "claims" provide "notice" of what in the patent constitutes the "new" and patentable invention, as opposed to the "old" and nonpatentable material surrounding it. As this Court explained, the 1836 Act "very wisely require[d] of the applicant a distinct and specific statement of what he claims to be new" since "in every application for a patent, the descriptive part is necessarily largely occupied with what is not new." *Merrill*, 94 U.S. at 570. The Patent Act of 1870 carried forward that purpose, requiring the inventor to "particularly point out and distinctly claim the part, improvement, or combination which he claims as his invention or discovery." Act of July 8, 1870, ch. 230, § 26, 16 Stat. 198. And the 1952 Patent Act, which states that a patent's specification shall include "one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention," 35 U.S.C. § 112, "is not materially different from the 1870 Act with regard to claiming, reissue, and the role of the PTO," *Warner-Jenkinson Co.* v. *Hilton Davis Chem. Co.*, 520 U.S. 17, 26 (1997).

The Patent Act imposes additional requirements that add to the concreteness and clarity of patents. For ex-

ample, the specification must contain "a written description of the invention, and of the manner and process of making and using it." 35 U.S.C. §112; see *Ariad Pharms., Inc.* v. *Eli Lilly & Co.*, 598 F.3d 1336, 1344 (Fed. Cir. 2010) (en banc). That description must be "in such full, clear, concise, and exact terms *as to enable any person* skilled in the art * * * *to make and use the [invention].*" 35 U.S.C. §112 (emphasis added). That requirement is known as "enablement." See *CFMT, Inc.* v. *YieldUP Int'l Corp.*, 349 F.3d 1333, 1338 (Fed. Cir. 2003).

In *Markman*, this Court held that the construction of patent claims is "an issue for the judge not the jury." 517 U.S. at 391. As a result, federal courts now conduct specialized "*Markman*" hearings to determine the scope of patent claims. Because the question of what a claim means is inextricably bound up with the issue of indefiniteness—the notion that the claim does not sufficiently set forth what it covers—the Federal Circuit has recognized that indefiniteness "is a matter of claim construction," *Praxair, Inc.* v. *ATMI, Inc.*, 543 F.3d 1306, 1319 (Fed. Cir. 2008), to which "general principles of claim construction apply," *Young* v. *Lumenis, Inc.*, 492 F.3d 1336, 1346 (Fed. Cir. 2007). The Federal Circuit thus has ruled that claims are indefinite unless "those skilled in the art would understand what is claimed." *Ibid.*

II. Proceedings Below

A. The Invention

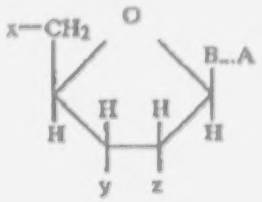
Labeled nucleotides are used in a variety of applications, including DNA sequencing and diagnosing hereditary diseases. Because each DNA sequence "pairs," "binds," or "hybridizes" predictably with the complementary DNA sequence (so AA-TT hybridizes with TT-AA), labeled nucleotides can be used to detect, monitor, localize, or isolate specific nucleic acid sequences (*e.g.*, DNA

sequences), even in small quantities. Pet. App. 3a. For example, a laboratory seeking a particular DNA sequence could introduce a so-called "probe" of the complementary DNA sequence made of labeled nucleotides. Any unhybridized material is later removed. The laboratory then looks for the labeled nucleotides. If they remain—having hybridized with the complementary DNA sequence—that indicates that the DNA sequence being tested for was present in the sample. See *ibid.*

Traditionally, labeled nucleotides were prepared by radioactive methods—*i.e.*, nucleotide sequences were marked by exposing them to radioactive materials, making them radioactive as well. While that made it possible to "see" them, it had many drawbacks. Radioactive nucleotides are potentially hazardous, and thus expensive and inconvenient to transport, use, and store. Pet. App. 3a-4a.

Dr. David Ward and co-workers at Yale University revolutionized the field by preparing chemically-labeled DNA molecules that still function like DNA. Previously, chemical labels (*e.g.*, fluorescent dyes) were not considered acceptable for marking DNA because it was generally assumed that structural modification to incorporate such a label would compromise the molecule's ability to function as DNA. But Dr. Ward synthesized a series of novel nucleotide derivatives that solved the problem. As one observer described it, his invention "can light up our genes in a kaleidoscope of colours and make them glow," an innovation involving "the physics of optics, computational science, digital imaging, microscopy, molecular biotechnology and cell biology." *Oration Honouring David Christian Ward* (1999), *available at* http://www.mun.ca/president/99-00report/honor/honorary_ward.html. For that groundbreaking invention, Dr. Ward was inducted into the National Academy of Sciences.

This case concerns patents arising out of that path-marking invention. U.S. Patent No. 5,449,767 ("the '767 Patent") sets forth one aspect of Dr. Ward's invention. It defines "an oligo- or polynucleotide containing a nucleotide" of the following structure:



'767 Patent col. 3, C.A. App. 169 (App., *infra*, 6a). The central 5-member ring is a sugar "moiety" or segment. "B" denotes a base (either a 7-deazapurine or a pyrimidine) attached to a specific place on the sugar ring. "A" denotes the label (*e.g.*, the dye). The label A is attached to the base B at a specific position defined by the claim. The patent describes the invention—and the nature and properties of each element in the diagram—at length. The general description of the invention spans two pages, and the detailed description of the invention spans twelve. See '767 Patent cols. 3-30, C.A. App. 169-182 (App., *infra*, 6a-19a). To design labeled DNA outside the claimed invention's scope, one would simply attach the label to a different, unclaimed position on the base B or to the sugar.

In questioning the patent's definiteness, petitioners do not contend that the base, the sugar, or the label are inadequately defined. Instead, they attack a small piece of the molecule, the "linkage group" (depicted as a dotted line in the formula) that connects the base and label. Per the claim, the linkage group "does not substantially interfere with the characteristic ability of the oligo- or polynucleotide to hybridize with a nucleic acid." See '767 Patent col. 31, C.A. App. 183 (App., *infra*, 31a). The petition urges that the description does not sufficiently

define "substantially" to allow someone to avoid infringement. But the petition nowhere explains why anyone would want to circumvent the patents by designing a linkage group that *does* interfere with the hybridization process. Such a molecule would be useless in any application that requires the modified DNA to function as "natural" DNA, *i.e.*, it would be contrary to the invention's very purpose of producing labeled DNA that functions just like unlabeled DNA.

The PTO specifically considered virtually indistinguishable claim language. Pet. App. 16a. The '767 Patent issued from a chain of applications, including Application Serial No. 496, 415. In a March 21, 1986 Supplemental Amendment, the applicants claimed a "linkage group not interfering substantially with the characteristic ability of A to form a stable complex with avidin." C.A. App. 4270-4271 (App., *infra*, 24a, 25a, 28a). The examiner initially rejected that language as "indefinite," and requested a structural definition. *Id.* at 4284 (App., *infra*, 38a). In response, the applicants explained why structural language would not adequately define their invention. *Id.* at 4306-4307 (App., *infra*, 40a-44a). Agreeing, the examiner "withdrew the indefiniteness objection," Pet. App. 16a, and the next office action limited indefiniteness concerns to misspellings and other formalities. *Id.* at 4323 (App., *infra*, 47a).

B. The District Court's Decision

Following a *Markman* hearing, the district court construed the disputed "linkage group" language along with other claims language. Pet. App. 7a, 39a.[2] Petitioners then moved for summary judgment, asserting that the invention was anticipated by prior art and failed to

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[2] The claims construction ruling is not included in the petition appendix but is publicly available as No. 3:04cv929 (JBA), 2006 U.S. Dist. Lexis 74570 (D. Conn. Oct. 12, 2006).

comply with the written description, enablement, and claim definiteness requirements of 35 U.S.C. § 112. Pet. App. 35a.

The court denied summary judgment on petitioners' enablement and written description defenses, finding disputed issues of material fact. Pet. App. 40a-51a. The district court invalidated some claims as anticipated by prior art. *Id.* at 61a-73a. Finally, the court found Dr. Ward's "linkage group" indefinite based primarily on the views of petitioners' expert, rejecting the contrary views of respondents' expert. *Id.* at 55a-58a. Ignoring Dr. Ward's extensive guidance for selecting linkage groups, the district court looked instead for an exact, numerical standard of "substantial interference." *Id.* at 54a-61a. Finding none, the court held the patents indefinite.

C. The Federal Circuit's Decision

The Federal Circuit affirmed in part and reversed in part. Turning to indefiniteness first, the court of appeals unanimously held that the claim terms were sufficiently definite. Pet. App. 10a-18a.

The court explained that resolving an "indefiniteness" claim "requires a determination whether those skilled in the art would understand what is claimed." Pet. App. 11a (quoting *Young* v. *Lumenis, Inc.*, 492 F.3d 1336, 1346 (Fed. Cir. 2007)). In this case, the court explained, one skilled in the art would understand the meaning of the phrase "not interfering substantially" because, among other things, there were dependent claims in the patent that identified specific linkage groups. Pet. App. 14a. Citing longstanding precedent, the court explained that a person of ordinary skill in the art would understand "the term 'not interfering substantially'" in the challenged claims as "allow[ing] for at least as much interference as that exhibited" using the particular "linkage group * * * specified in the dependent claims." *Ibid.* Additional ex-

amples of linkage groups were found elsewhere in the specification, along with "criteria for selecting among them." *Id.* at 15a. Dr. Ward provided examples of tests performed on particular linkage groups, identifying specific results that were deemed "acceptable." *Id.* at 15a-16a. Those examples also taught that a polynucleotide's "thermal denaturation profile" (essentially, a comparison of the melting point of labeled hybrids to unmodified material) can be used to measure the degree to which a linkage group interferes with hybridization. *Id.* at 15a. Finally, looking to the patent's "prosecution history," the decision below found still more examples of linkage groups that did not substantially interfere. *Id.* at 16a.

The court did not mention the extrinsic testimony the district court cited as evidence of indefiniteness. Instead, it ruled that "the intrinsic evidence here provides 'a general guideline and examples sufficient to enable a person of ordinary skill in the art to determine [the scope of the claims],' *In re Marosi*, 710 F.2d 799, 803 (Fed. Cir. 1983)." Pet. App. 16a-17a (brackets in original). A "person of ordinary skill in the art," the court concluded, could "look to the thermal denaturation and hybridization (including T_m) profiles to see if they fall within the range of exemplary values disclosed in the intrinsic evidence." *Id.* at 17a.

The Federal Circuit was not asked to pass on, and did not pass on, any dispute regarding proper construction of the "linkage group." Nonetheless, the court *sua sponte* modified that construction as to one of the patents, and on that basis, found it anticipated by prior art. Pet. App. 13a, 26a-27a. As to the other two Ward patents, the court found disputed issues of material fact, reversed summary judgment on anticipation grounds, and "remanded for further proceedings consistent with" its opinion. *Id.* at 21a-26a, 33a.

Although Judge Plager joined the panel's unanimous opinion, he dissented from denial of rehearing en banc. Pet. App. 75a-79a. No other judge joined him.

REASONS FOR DENYING THE PETITION

The decision below does not conflict with any decision of this Court, PTO examination practice, or any statutory text. The assertion of importance is unfounded. The case's interlocutory posture is by itself reason for denying review in favor of a case with a fully developed record. And the case in any event does not properly present the question on which petitioners seek review.

I. The Decision Below Does Not Conflict With Any Decision Of This Court Or Statutory Text

Petitioners' assertion of conflict is based on a mistaken premise. This Court has never held that patent claims must mark the exact boundaries separating the invention from all potential infringements. Rather, the law requires only the certainty that is "reasonable" under the circumstances. This Court thus has upheld as "sufficient" expressions "quite as indefinite as 'high' and 'substantial,' in describing an invention or discovery, in patent specifications and claims." *Eibel Process Co.* v. *Minn. & Ontario Paper Co.*, 261 U.S. 45, 66 (1923).

The Court has required the patent to define the boundaries between the claimed invention and other, non-novel subject matter disclosed in the application. But the Court has never suggested that claims must also chart safe harbors for potential infringers by describing in detail everything the invention is not. Petitioner's contrary view—that patent claims cannot brook any ambiguity—cannot be reconciled with this Court's precedents. It defies this Court's longstanding requirement that courts "construe" a claim with two possible meanings by adopting a construction that preserves the inventor's rights. And it is inconsistent with this Court's

adherence to the doctrine of equivalents which, by definition, affords patentees rights beyond the literal claims of a patent.

A. The Federal Circuit Correctly Applied This Court's Precedents Permitting Relative Terms Like "Substantially"

1. Petitioners and their *amici* ignore this Court's longstanding recognition that "the certainty which the law requires in patents is not greater than is reasonable, having regard to their subject matter." *Minerals Separation* v. *Hyde*, 242 U.S. 261, 270 (1916). Consistent with that principle, inventors may assert claims using "[e]xpressions quite as indefinite as 'high' and 'substantial.'" *Eibel Process*, 261 U.S. at 66. To avoid indefiniteness, the inventor need not provide an exact description. Instead, given the "large class of substances and the range of treatment within the terms of the claims," it is permissible for inventors to "leav[e] something to the skill of persons applying the invention" so long as the claims are "sufficiently definite to guide those skilled in the art to its successful application." *Mineral Separation*, 242 U.S. at 271.

In this case, the Federal Circuit followed that formula. Resolving an "indefiniteness" claim, the court noted, requires "a determination whether those skilled in the art would understand what is claimed." Pet. App. 11a (quoting *Young* v. *Lumenis, Inc.*, 492 F.3d 1336, 1346 (Fed. Cir. 2007), in turn quoting *Datamize, LLC* v. *Plumtree Software, Inc.*, 417 F.3d 1342, 1348 (Fed. Cir. 2005)). Ignoring the standard actually applied below, petitioners assail the notion that the claim must be "insolubly ambiguous" to be indefinite. See Pet. i, 3, 4, 15, 19, 23. But that gloss is never mentioned by the decision below. Besides, any gloss must take meaning from the standard applied below and this Court's cases—*i.e.*, the claim is "insolubly ambiguous" only when "those

skilled in the art," upon examining the claim in light of their knowledge and other disclosures in the patent, "would [not] understand what is claimed."

The court of appeals properly applied those principles. Although the claim itself uses relative terms ("substantially interferes") in describing unacceptable linkage groups, the court identified disclosures throughout the patent—including "dependent claims," examples and discussion in the patent's specification, and particular examples of suitable linkages in the prosecution history—that would allow one skilled in the art to determine the permissible degree of interference. See pp. 10-11, *supra*. The patent, the court held, would "allow for at least as much interference as that exhibited" by the identified "linkage group[s]." Pet. App. 14a; see also 15a-16a. This Court's cases regularly follow a similar course, evaluating the claim in light of the rest of the patent. See *Eibel Process*, 261 U.S. at 65-66; *United Carbon Co.* v. *Binney & Smith Co.*, 317 U.S. 228, 235-236 (1942); *Gen. Elec. Co.* v. *Wabash Appliance Corp.*, 304 U.S. 364, 373 (1938).

Petitioners overlook longstanding precedent allowing inventors to claim with relative words of degree like "substantially." The term "maintained at a substantial elevation" has been sustained against an indefiniteness challenge. *Eibel Process*, 261 U.S. at 65. Claims to a rubber-curing process including the step of "heating said mold to a temperature range approximating a predetermined rubber curing temperature" were held patentable. *Diamond* v. *Diehr*, 450 U.S. 175, 181 (1981). And Alexander Graham Bell's pioneering telephony patent, which claimed "[t]he method of, and apparatus for, transmitting vocal or other sounds telegraphically, as herein described, by causing electrical undulations, *similar in form to* the vibrations of the air," was upheld. *Dolbear* v. *Am. Bell Tel. Co.*, 126 U.S. 1, 163 (1888)

(emphasis added). So too were claims to "the manufacturing of fat acids and glycerides from fatty bodies by the action of water at a high temperature and pressure." *Tilghman* v. *Proctor*, 102 U.S. 707, 721 (1880). In none of those cases did the relative claim language fix the "metes and bounds" of the invention, as petitioners would demand. See Pet. 2. Instead, the claims would have to be construed in light of the rest of the patent.[3]

Petitioners' opposition to relative terms, moreover, is inconsistent with established PTO practice. The PTO's Manual of Patent Examining Procedure ("MPEP") instructs examiners to reject "relative" terms like "substantially" as indefinite *only* when "the specification does not provide a standard for ascertaining the requisite degree, and one of ordinary skill in the art would not be reasonably apprised of the scope of the invention." MPEP § 706.03(d) (8th ed. 2004). That instruction comports with the Federal Circuit's approach here: The court upheld the use of the relative term "substantially interfere" because the specification (including dependent claims) allowed one of ordinary skill in the art to determine the tolerable degree of interference. That sort of careful, context-specific analysis pervades similar Federal Circuit cases.[4]

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[3] While *Diehr*, *Dolbear*, and *Tilghman* did not address indefiniteness, this Court regularly resolves patent cases on grounds other than those raised by the parties. See *Permutit Co.* v. *Graver Corp.*, 284 U.S. 52 (1931) (petition raising novelty issues resolved on indefiniteness grounds); *Dann* v. *Johnston*, 425 U.S. 219 (1976) (addressing "obviousness" where question presented was directed to the patentability of the subject matter).

[4] See, *e.g.*, *Andrew Corp.* v. *Gabriel Elecs., Inc.*, 847 F.2d 819 (Fed. Cir.) ("substantially"), cert. denied, 488 U.S. 927 (1988); *Quantum Corp.* v. *Rodime, PLC*, 65 F.3d 1577 (Fed. Cir. 1995) ("approximately"), cert. denied, 517 U.S. 1167 (1996); *Exxon Research & Eng'g* v. *United States*, 265 F.3d 1371 (Fed. Cir. 2001) ("substan-

Petitioners' view also defies other settled principles. For example, the Court's decision in *Markman* teaches that ambiguous claim terms must be construed by the court. 517 U.S. at 387-388. Correspondingly, *Smith* teaches that courts should adopt constructions that save rather than invalidate the patent so as to "secure to the patentee his actual invention." 294 U.S. at 14. Such efforts would be pointless if the ambiguity that necessitated the act of construction would render the patent indefinite in any event, as petitioner assumes and one of its *amici* insists. See Pet. 18-19; Altera Br. 10-11.[5] Finally, petitioners' view cannot be reconciled with *Festo Corp.* v. *Shoketsu Kinzoku Kogy Kabushiki Co.*, 535 U.S. 722, 732 (2002), which upholds the "doctrine of equivalents." Under that doctrine, "the scope of the patent is not limited to its literal terms but instead embraces all equivalents to the claims described." *Ibid.* While that necessarily "renders the scope of patents less certain," this Court has recognized that it represents "the price of ensuring the appropriate incentive for innovation, and has affirmed the doctrine over dissents that urged a more certain rule." *Ibid.* *Festo* forecloses petitioners' view that the "claims" language must mark the precise boundaries of the inventor's rights.

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tial"); *Deering Precision Instruments, LLC* v. *Vector Distribution Sys., Inc.*, 347 F.3d 1314 (Fed. Cir. 2003) ("substantially"), cert. denied, 540 U.S. 1184 (2004); *Ortho-McNeil Pharm., Inc.* v. *Caraco Pharm. Labs., Ltd.*, 476 F.3d 1321 (Fed. Cir. 2007) ("about").

[5] Petitioner's suggestion that Federal Circuit practice allows vague constructions to substitute for vague claim language is not well-founded. That court can and has invalidated patents where the construction is vague. See, *e.g.*, *Halliburton Energy Servs., Inc.* v. *M-I LLC*, 514 F.3d 1244, 1251 (Fed. Cir. 2008) ("Even if a claim term's definition can be reduced to words, the claim is still indefinite if a person of ordinary skill in the art cannot translate the definition into meaningfully precise claim scope.").

The Federal Circuit's longstanding approach to indefiniteness is thus firmly grounded in this Court's cases. It is therefore little surprise that, for at least a decade, the Federal Circuit has applied its settled standards in multiple cases heard by multiple panels without a single dissent by an active judge. Judge Plager's dissent from denial of rehearing en banc appears to be a solitary view.

2. Petitioners' contrary theory, moreover, derives from a misreading of this Court's precedents. According to petitioners, patent claims must clearly distinguish the invention from anything and everything not infringing. But petitioner cannot identify a *single case* in which this Court has invalidated claims based on indefiniteness because the claims, although capable of reasonable construction, did not provide potential infringers with a blueprint for designing around the patent. Instead, petitioners' cases address patents that mixed the new invention with pre-existing machines or inventions and failed to distinguish adequately between the claimed innovation and the "old" or pre-existing art.

For example, in *Wabash Appliance*, the Court observed that Congress, "[r]ecognizing that most inventions represent improvements on some existing article, process, or machine, and that the description of the invention must in large part set out what is old in order to facilitate the understanding of what is new," appropriately "require[d] of the applicant 'a distinct and specific statement of what he claims to be new, and to his invention.'" 304 U.S. at 368-369. The patent before it, the Court held, failed to "adequately set out 'what he claims to be new'" because its description of the invention also "serve[d] aptly to describe the product of earlier manufacture." *Id.* at 369-370. "The failure of the patentee to make claim to a distinct improvement" over the prior art therefore doomed the claim. *Id.* at 370. Similarly, in *United Carbon*, the Court was concerned about "the

somewhat indeterminate character of the advance claimed to have been made" because the prior art patents "describe[d] products" that, "if not identical" to the claimed invention, were "at least of confusing similarity." 317 U.S. at 237.

Finally, the claims in *Permutit Co.* v. *Graver Corp.*, 284 U.S. 52 (1931), were not even ambiguous. Instead, the Court found the disclosures inadequate because, while the inventor claimed his invention improved on prior art by using a "free" zeolite bed combined with "rising space" above the bed, the patent's claims did not mention either feature. *Id.* at 57. The Court acknowledged that drawings attached to the patent showed both features, and that such "drawings * * * may be used as an aid to interpreting the specification or claim," but it concluded that they were "of no avail" there given the "entire absence of description of the alleged invention or a failure to claim it." *Id.* at 60. Here, by contrast, every feature of the claimed invention is set forth in the claims, and the patent's specification resolves any ambiguity.

B. The Federal Circuit's Definiteness Standard Does Not Conflict With The Patent Statute

Petitioners also assert (at 16-18) that the Federal Circuit's standard is inconsistent with the Patent Act's requirement that the specification contain "a written description of the invention" and "one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention." 35 U.S.C. § 112. But petitioners ignore the origins of those requirements. The claim must "particularly point out" what the invention *is* by distinguishing the invention from other subject matter disclosed in the written description. A claim that is subject to construction does precisely that. The statute does not require the patent to delineate what the claim *is not* so as to distinguish the invention from every conceivable potential infringement.

Here, a person skilled in the art would fully have understood the nature of the invention.

1. As explained in greater detail above, see pp. 4-6, *supra*, the language in §112 derives from a series of decisions of this Court insisting *not* that the patent set forth a blueprint for circumvention but rather that it distinguish the new invention being claimed from preexisting art. This Nation's first two patent laws (the Patent Acts of 1790 and 1793) required inventors to "distinguish" their inventions from "other things before known," but made no mention of "claims" per se. See Act of Apr. 10, 1790, ch. 7, §2, 1 Stat. 109; Act of Feb. 21, 1793, ch. 11 §3, 1 Stat. 318. Instead, claims practice derived from *Evans* v. *Eaton*, 20 U.S. 356 (1822), which addressed whether subject matter must be identified as inventive, or whether instead novelty "may be established by comparing the machine specified in the patent with former machines in use." *Id.* at 433; see 3 *Chisum on Patents* §8.02[1] (2009). Construing the 1793 Patent Act, the Court held that the Act did not authorize a patent "[w]hich describes the machine fully and accurately, as a whole, mixing up the new and old, but does not *in the slightest degree* explain what is the nature or limit of the improvement which the party claims as his own." 20 U.S. at 433 (emphasis added).

Since then, this Court's cases have recognized that, because patents representing "improvements on some existing article" often must describe the new improvement together with the preexisting device, inventors must distinctly and specifically state what they "claim[ ] to be new." *Wabash*, 304 U.S. at 378-379. That requirement was incorporated into the Patent Act of July 4, 1836, ch. 357, §6, 5 Stat. 119, see *Markman*, 517 U.S. at 379, which required the inventor to "particularly specify and point out the part, improvement, or combination which he claims as his own invention or discovery." The

Court thus explained how the 1836 Act "very wisely require[d] of the applicant a distinct and specific statement of what he claims to be new" since "in every application for a patent, the descriptive part is necessarily occupied with what is not new." *Merrill*, 94 U.S. at 570. Similar language appeared in the 1870 Act. See p. 5, *supra*. And the 1952 Patent Act, which placed the claim definiteness requirement in its current form, "is not materially different from the 1870 Act with regard to claiming." *Warner-Jenkinson*, 520 U.S. at 26. The Act thus requires "notice" of what the invention "is" in the sense of distinguishing it from preexisting machines or prior art also described in the patent. But there is no requirement that it specify all means of infringement.

2. Petitioners' contrary arguments are premised almost entirely on the court-made doctrine that the claims "define" the invention. Pet. 16.[6] As an initial matter, that doctrine has never had the absolute quality petitioners attribute to it. For example, the doctrine of equivalents gives inventors rights beyond the literal terms of the claims. See p. 3, *supra*. Under that doctrine, "a product or process that does not literally infringe upon the express terms of a patent claim may nonetheless be found to infringe if there is 'equivalence' between the elements of the accused product or process and the claimed elements of the patented invention." *Warner-Jenkinson*, 520 U.S. at 25-26; cf. *Winans* v. *Denmead*, 56 U.S. 330, 342 (1853) (infringement not limited to "the precise forms * * * described, but all other forms which embody [the] invention."). This Court has speci-

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[6] The statutory definition of infringement, 35 U.S.C. § 221, did not even mention claims until 2003, when a declaratory judgment remedy was created for generic drug makers. See 35 U.S.C. § 271(e)(5); *Teva Pharms. USA, Inc.* v. *Pfizer, Inc.*, 395 F.3d 1324, 1334 (Fed. Cir. 2005).

fically rejected the notion that the doctrine of equivalents is inconsistent with § 112's requirement that the invention be identified with appropriate clarity. See *Warner-Jenkinson*, 520 U.S. at 25-26. That doctrine forecloses the notion that the claims themselves must specifically identify all infringing activities.

Besides, even if the claims "define" the invention, this Court has not confined its infringement analysis to the claims language. This Court regularly looks to other portions of the patent when construing claim terms. *United States* v. *Adams*, 383 U.S. 39, 49 (1966) ("[I]t is fundamental that claims are to be construed in the light of the specifications and both are to be read with a view to ascertaining the invention."); *White* v. *Dunbar*, 119 U.S. 47, 60 (1886) (looking to "the context of the specification" to determine whether it supported "giv[ing] the claim so broad a construction"); *Permutit Co.*, 284 U.S. at 60 (attached "drawings * * * may be used as an aid to interpreting the * * * claim"). Prior art may help define the claim, counseling a relatively broader or narrower construction. *Eibel Process*, 261 U.S. at 63; *Smith*, 294 U.S. at 14 ("the character of the patent and its commercial and practical success are such as to entitle the inventor to broad claims and to a liberal construction of those which he has made"). Infringement too may be judged by reference to "the mechanism described in the specification." *Fuller* v. *Yentzer*, 94 U.S. 288, 293 (1876). The decision below did no more than what this Court's cases allow—holding the relevant claims language, when read in connection with the remainder of the patent, sufficiently definite to guide one reasonably skilled in the art.

C. There Is No Conflict Between The Federal Circuit And The PTO

Petitioners assert (at 14-15) a conflict between the Federal Circuit's standard of indefiniteness and the

standard used by the PTO's Board of Patent Appeals ("BPAI") in processing patent applications. Petitioners note that the BPAI may "requir[e] the applicant to more precisely define the metes and bounds of the claimed invention by holding the claim unpatentable" when "a claim is amenable to two or more plausible claim constructions." Pet. 14 (internal quotation marks omitted). But petitioners overlook *why* the PTO will issue rejections under that more expansive standard. As the Board explained in *Ex Parte Miyazaki*, 89 U.S.P.Q.2d 1207 (BPAI 2008), the PTO applies a different standard in the application process "because the applicant has *an opportunity* and a duty *to amend the claims during prosecution* to more clearly and precisely define the metes and bounds of the claimed invention." *Id.* at 1211-1212 (emphasis added). But once the patent has been issued and infringement is being litigated, there is no opportunity to amend. For courts, the options are enforcing the patent or invalidating it.

The PTO's decision to apply a different standard thus does not reflect a disagreement with the Federal Circuit or a different construction of §112. It reflects the fact that, when the application is before the PTO, "claims can be amended, ambiguities should be recognized, scope and breadth of language explored, and clarification imposed." *In re Zletz*, 893 F.2d 319, 321 (Fed. Cir. 1989). The Federal Circuit likewise has never faulted the PTO's standard. "It would be inconsistent with the role assigned to the PTO in issuing a patent," the Federal Circuit has observed, "to require it to interpret claims in the same manner as judges who, post-issuance, operate under the assumption the patent is valid." *In re Morris*, 127 F.3d 1048, 1054 (Fed. Cir. 1997). Because the "process of patent prosecution is an interactive one," examiners may insist on amendments to obtain the greatest clarity

possible. *Ibid.* But judicial decisions on validity are binary—the patent is either invalidated or upheld. *Ibid.*

Indeed, the PTO and the Federal Circuit regularly apply different standards in myriad contexts in light of their different roles:

- Before a patent issues, the PTO will give claims their "broadest reasonable interpretation" in light of the specification, MPEP, *supra*, §2111, to force applicants to amend them by narrowing and clarifying, *In re Zletz*, 893 F.2d at 321. "During litigation determining validity or infringement, however, this approach is inapplicable." *Atlantic Thermoplastics Co.* v. *Faytex Corp.*, 970 F.2d 834, 846 (Fed. Cir. 1992). Instead, courts give claims their most natural construction. *Ibid.*

- Before a patent issues, patent examiners who make out a *prima facie* case of obviousness will assign the applicant the burden of *disproving* obviousness. See MPEP, *supra*, §2131. After a patent issues, the burden of proving the patent "obvious" in litigation always remains on the party challenging the patent's validity. See *Sanofi-Synthelabo* v. *Apotex, Inc.*, 550 F.3d 1075, 1086 (Fed. Cir. 2008).

- Before a patent issues, the PTO can require an applicant invoking commercial success as evidence of non-obviousness to provide hard evidence of success and a nexus to novelty. See MPEP, *supra*, §2145. Once the patent is issued, a *prima facie* case is established whenever a successful product is claimed in the patent, and the opposing party has the burden of disproving it. See *Demaco Corp.* v. *F. Von Langsdorff Licensing Ltd.*, 851 F.2d 1387, 1392 (Fed. Cir. 1988).

- Before a patent issues, the PTO can force the applicant to provide a "satisfactory showing" that its claim of inventorship is correct if a publication names inventors other than those identified in the application. *In re Katz*, 687 F.2d 450, 455 (C.C.P.A. 1982). After a patent issues, the party alleging misjoinder must prove it "by clear-and-convincing evidence." See *Eli Lilly & Co.* v. *Aradigm Corp.*, 376 F.3d 1352, 1358 (Fed. Cir. 2004).

- Before a patent issues, a specification must be accepted as compliant with the enablement requirement of § 112, "unless there is reason to doubt the objective truth of the statements contained therein." *In re Marzocchi*, 439 F.2d 220, 223 (C.C.P.A. 1971). After a patent issues, the accused infringer is free to dispute whether the specification enables. See *Amgen, Inc.* v. *Chugai Pharm. Co., Ltd.*, 927 F.2d 1200 (Fed. Cir. 1991).

Petitioners' charge of "conflict" thus ignores the fact that PTO examination standards and judicial enforcement standards often diverge, and properly so, in light of the PTO's and the courts' distinct roles; the fact that the PTO can and should require amendment, while courts can only invalidate; and the fact that no presumption of validity attaches to a patent application before the PTO, but does attach once the patent issues.

There is no conflict, moreover, between the PTO and the Federal Circuit on the proper result here. The PTO, like this Court and the Federal Circuit, recognizes that relative terms like "substantial" are permissible so long as sufficient guidance can be discerned from the specification. See p. 15, *supra*; MPEP, *supra*, § 706.03(d). And both the PTO and the Federal Circuit agreed that the standard of definiteness was met here. The PTO examiner approved the application here only after his

concerns about the definiteness of virtually identical language were overcome. See Pet. App. 16a; p. 9, *supra*. The Federal Circuit, examining the same materials presented to the examiner, likewise agreed that the standard of definiteness was met. Pet. App. 13a-16a. This Court can hardly resolve a supposed conflict between two bodies on the standard of definiteness in a case where both bodies reached the identical conclusion that the definiteness requirement was met. This Court "'reviews judgments, not statements in opinions.'" *California* v. *Rooney*, 483 U.S. 307, 311 (1987) (quoting *Black* v. *Cutter Labs.*, 351 U.S. 292, 297 (1956)).

II. The Request For Further Review Falls Short of Justification

Petitioners have failed to demonstrate that this issue is important. For a decade, the Federal Circuit has employed its current standards without meaningful dispute or dissent. Before addressing petitioners' specific claims, however, we pause to point out precisely what petitioners are asking the Court to do. For almost a century, this Court has authorized the use of "[e]xpressions quite as indefinite as 'high' and 'substantial.'" *Eibel Process*, 261 U.S. at 66. In reliance on that and other precedents, patent applicants routinely seek and obtain patents with claim language like "substantially," "about," and "approximately"—terms that necessarily do not have a definite meaning standing by themselves, but rather gather their meaning from the context of the rest of the patent, such as the written description, the specification, charts and figures, working and/or prophetic examples, and descriptions of specific embodiments.

The PTO allows the use of such terms when the surrounding materials are sufficient. Indeed, even petitioners and their *amici* have relied on them. Since 1976, Applera has been assigned 102 patents that use the term "substantially," Yahoo has 25, Google has 34, Cisco Sys-

tems or its subsidiary has 479, Microsoft has 758, and General Motors has 3,047.[7] That word, "substantially," necessarily requires construction: It can "denote either language of approximation or language of magnitude" and does not in and of itself answer *how close* or *"how much."* Pet. App. 13a, 14a. *Amici* can urge that all those patents "have been prosecuted in compliance with the Patent Act" nonetheless, Altera Br. 1, only because nothing in the Patent Act imposes on inventors the draconian standards of definiteness petitioners advocate. In any event, given the overwhelming reliance on this Court's approval of relative claim terms in cases like *Eibel Process*, *stare decisis* weighs heavily against an effort to overturn them. See pp. 2, 14-15, *supra*. Such a tectonic upheaval in the Court's construction of a statute should not be contemplated absent a compelling need.

Petitioners have established no such need. Notwithstanding decades of application by myriad panels in myriad cases, the Federal Circuit's standards drew no serious dissents until Judge Plager's lone dissent in this case. That renders any claim of importance highly suspect. *Amici* cite an article quoting anonymous "experts in claim drafting" for the proposition that claims can be vaguely worded intentionally. Altera Br. 13. But *amici* do not show that the advice of those unidentified "experts" is followed. Nor should it have any effect: The PTO has authorized its examiners to *reject* any claim amenable to two or more constructions. *Ex Parte Miyazaki*, 89 U.S.P.Q.2d at 1209. Here, moreover, the applicants did not choose the disputed claim language to render the patent vague. As they explained to the PTO's

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[7] Based on the Advanced Search feature of the PTO's website as of November 4, 2009. See http://patft.uspto.gov/netahtml/PTO/search-adv.htm.

satisfaction, the language was the most accurate definition of the invention possible. See p. 9, *supra*.

Asserting that the Federal Circuit's standard materially alters litigation outcomes, *amici* characterize one paper as showing that, in district court, patentee success rates on indefiniteness challenges "increased by sixty percentage points" between 1998 and 2008. Altera Br. 11 (citing Christa J. Laser, *A Definite Claim on Claim Indefiniteness*, 11 Chi.-Kent J. Intell. Prop. 25 (2010)). But *amici* fail to mention that the same article found that patentee success rates before the Federal Circuit fell precipitously in the last data point of the article's already small data set. Laser, *supra*, at 33, Fig. 2. That finding undermines their supposed "trend." Far from being "toothless," the definiteness requirement remains a powerful tool that has disposed of substantial infringement cases. See, *e.g.*, *Acacia Media Techs. Corp.* v. *New Destiny Internet Group*, No. 05-CV-114, MDL Case No. 05-1665, (N.D. Cal. Oct. 23, 2009) (granting summary judgment in multi-district case involving five patents and 45 defendants), *aff'd*, No. 2010-1081 (Fed. Cir. Oct. 7, 2010); *Quantum Corp.*, 65 F.3d at 1580-1584. And the assault on suits filed by non-practicing entities ("NPEs") is baseless. In the vast majority of cases taken to judgment, the accused infringer wins. Altera Br. 16 n.6.[8] Any NPE "problem" should be remedied by, for example, liberalizing the Patent Act's fee-shifting provision, 35 U.S.C. §285, not by radically changing the law of definiteness to the detriment of Nobel laureates, National Academy inductees, and other contributors to the useful arts and sciences.

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[8] Like all research universities, respondent Yale University is expanding knowledge rather than selling products. NPEs like Yale have made countless contributions to the useful arts and sciences, including the invention asserted in the Ward patents.

Finally, *amici* urge that the Federal Circuit's reversal rate on claims-construction issues proves that claims are excessively ambiguous because district courts allegedly "get[] it wrong nearly half the time." Altera Br. 8. But the reported reversal rates cannot account for the inherent selection bias resulting from rational litigants appealing only relatively close cases, leaving the cases where the district court has clearly "gotten it right" unappealed. Indeed, petitioners did just that here, declining to appeal the district court's construction of the linkage group.[9] The reversal rate may also reflect that claim construction "is a special occupation, requiring, like all others, special training and practice," *Markman*, 517 U.S. at 388, and the Federal Circuit's tendency, as a specialist court that reviews claims construction *de novo*, to see things differently than generalist district courts. In any event, petitioners have not demonstrated that the process is unworkable, that skilled judges cannot ascertain the scope of most patents, or that any issues that do arise are a product of patent quality as opposed to the immense scientific and technological complexity of many inventions. Nor have they shown that, to the extent any problem exists, it should be "solved" by invalidating more patents for indefiniteness as opposed to addressing the Federal Circuit's method of claims construction.

In any event, petitioners' unsubstantiated attempt to radically revise claims practice is misdirected. This Court decided *Eibel Process* in 1923, and Congress overhauled the patent laws in 1952. If Congress had thought the sort of claims language authorized by *Eibel*

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[9] *Amicus* Yahoo challenges the result in the *Bright Response* case. Yahoo Br. 14-16. While that may be an unfortunate example of a district court (actually a magistrate judge) "getting it wrong," one erroneous decision from a non-Article III judge hardly justifies this Court's intervention.

Process improper, it would have placed corrective language in the 1952 Act. See *Hilton*, 502 U.S. at 202. Far from doing so, Congress carried forward the relevant language of the 1870 Act into the 1952 Patent Act, which "is not materially different from the 1870 Act with regard to claiming, reissue, and the role of the PTO." *Warner-Jenkinson*, 520 U.S. at 26. If petitioners have a complaint about that decision, their remedy lies with Congress. Indeed, unlike this Court, Congress could legislate prospectively to avoid upsetting the public's reliance on this Court's longstanding acceptance of terms like "substantially" in appropriate contexts.

III. The Decision Below Is A Poor Vehicle

Finally, this case represents an inadequate vehicle for further review. First, notwithstanding petitioners' claim that the PTO and the Federal Circuit have adopted different standards for definiteness, both the PTO and the Federal Circuit agreed that these claims were sufficiently definite. The claims also passed muster with Judge Plager, who voted with the original panel that found them definite. His dissent from rehearing takes issue with the governing standard, but nowhere suggests that the claims here would be invalid under a plausible alternative.[10] Review premised on a purported disagreement between the PTO and the Federal Circuit should await a case in which they actually disagree. See pp. 24-25, *supra*. While petitioners also object (at 27-28) to certain "functional" claim language, that argument was neither pressed in district court nor addressed in the court of appeals. This Court is "a court of review, not of first view," and traditionally does not address issues that

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[10] Indeed, while claiming indefiniteness in the Federal Circuit, petitioners abandoned any effort to challenge the district court's construction of the claim. This case thus involves a claim with just one reasonable construction.

were not preserved or addressed below. *Cutter* v. *Wilkinson*, 544 U.S. 709, 719 n.7 (2005).[11]

The petition, moreover, does not properly present the issue it purports to raise. The invention here allows nucleotides to be "labeled" without interfering with their ability to function as DNA, *i.e.*, to "hybridiz[e]" in the same fashion as unlabeled DNA. It establishes a specific and concrete chemical structure to achieve that result. Thus, when first confronted with the allowed claims of the '767 Patent, petitioners sent a letter to the Assistant Commissioner of Patents demonstrating that they fully understood what the claims covered—"fluorescently labeled DNA sequencing fragments." C.A. App. 2793 (App., *infra*, 51a).

Petitioners nonetheless seize on one small part of the novel nucleotide derivative at issue, a "linkage" group that does not "substantially interfere" with hybridization. Pet. 9. According to petitioners, the patent fails to mark the boundaries of the invention because it does not clearly distinguish non-infringing nucleotides with linkage groups that *do* substantially interfere with hybridization from infringing molecules that do not. But petitioners never explain why anyone would deliberately label a nucleotide such that hybridization is obstructed; the whole point of the invention and the prior art of radiologically labeled nucleotides was to mark the nucleotides without affecting their ability to hybridize. Petitioners thus seek to invalidate a patent based on the notion that they cannot clearly ascertain a claims "boun-

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[11] The 1952 Patent Act specifically allows functional claim language, overruling the precedent petitioners cite. See *Valmont Indus., Inc.* v. *Reinke Mfg. Co., Inc.*, 983 F.2d 1039, 1042 (Fed. Cir. 1993). Had petitioners' objection been presented to the district court, it could have been addressed by construing the disputed language in accordance with the functional claiming provision of § 112.

dary" no reasonable person would approach. In essence, they assert that the patent is invalid because it does not tell them how to build around it by making a molecule that would not work.

Because patents are a quid-pro-quo in which the government awards an exclusivity period in exchange for disclosing the invention, *Kewanee Oil Co.* v. *Bicron Corp.*, 416 U.S. 470, 484 (1974), the Patent Act does require the patent to describe the invention and its use "in such full, clear, concise, and exact terms *as to enable any person* skilled in the art * * * *to make and use [it]*," 35 U.S.C. § 112 (emphasis added). If the patent does not "enable" others to build the invention, it is invalid. Pet. App. 14a n.4. But petitioners do not assert in this Court that the patent did not enable them to practice the invention. Instead, they assert that the patent is indefinite because the claim language, standing alone, does not clearly define a nucleotide with obstructed hybridization that would not work anyway.

Finally, the decision below is interlocutory—it merely remands the case for further proceedings. That alone is grounds for denying review, *Hamilton-Brown Shoe Co.* v. *Wolf Bros. & Co.*, 240 U.S. 251, 258 (1916), particularly in a case that, like this one, could use further factual development. On remand, petitioners presumably will present multiple challenges to the patents' validity, including challenges based on prior art. They may challenge enablement. As the foregoing discussion and petitioners' own submissions make clear, all of these issues are interrelated. See Pet. 22-23 n.9 (criticizing the Federal Circuit's holding that the defenses of anticipation and indefiniteness are mutually exclusive); Yahoo Br. 8-9 (arguing that some patent linkage group examples cited by the Federal Circuit are found in the prior art). If the question presented is to be reviewed at all, it should be reviewed in a case with a fully developed

record following a final judgment disposing of all related issues. And if this issue has the importance petitioners claim—and it does not—such a case will surely arise in due course. Review at this time is premature.

CONCLUSION

The petition for a writ of certiorari should be denied.

Respectfully submitted.

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November 2010

APPENDIX

US005449767A

United States Patent [19]

Ward et al.

[11] Patent Number: 5,449,767

[45] Date of Patent: * Sep. 12, 1995

[54] MODIFIED POLYNUCLEOTIDES AND METHODS OF PREPARING SAME

[75] Inventors: **David C. Ward**, Guilford, Conn.; **Penninn R. Langer**, Monsey, N.Y.; **Alexander A. Waldrop, III**, Charlottesville, Va.

[73] Assignee: **Yale University**, New Haven, Conn.

[*] Notice: The portion of the term of this patent subsequent to Dec. 8, 2004 has been disclaimed.

[21] Appl. No.: 886,660

[22] Filed: May 20, 1992

Related U.S. Application Data

[60] Continuation of Ser. No. 130,002, Dec. 8, 1987, abandoned, which is a division of Ser. No. 496,915, May 23, 1983, Pat. No. 4,711,955, which is a continuation-in-part of Ser. No. 255,223, Apr. 17, 1981, abandoned.

[51] Int. Cl.[6] C07H 21/00

[52] U.S. Cl. 536/24.3; 536/25.32; 536/25.6; 536/26.6

[58] Field of Search 536/24.3, 25.32, 25.6, 536/26.6; 435/6

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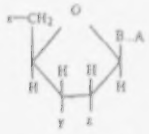
(List continued on next page.)

Primary Examiner—Che S. Chereskin
Attorney, Agent, or Firm—Ronald C. Fedus; John L. Santalone

[57] ABSTRACT

Compounds having the structure:

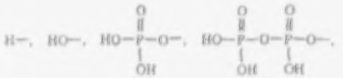


wherein B represents 7-deazapurine, or pyrimidine moiety covalently bonded to the $C^{1'}$-position of the sugar moiety, provided that when B is 7-deazapurine, it is attached at the N^9-position of the 7-deazapurine and when B is pyrimidine, it is attached at the N^1-position;

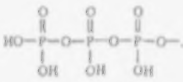
wherein A represents a moiety consisting of at least three carbon atoms which is capable of forming a detectable complex with a polypeptide when the compound is incorporated into a double-stranded ribonucleic acid, deoxyribonucleic acid duplex, or DNA-RNA hybrid;

wherein the dotted line represents a chemical linkage joining B and A, provided that if B is 7-deazapurine, the linkage is attached to the 7-position of the deazapurine, and if B is pyrimidine, the linkage is attached to the 5-position of the pyrimidine; and

wherein each of x, y and z represents



or



either directly, or when incorporated into oligo- and polynucleotides, provide probes which are widely useful.

71 Claims, No Drawings

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MODIFIED POLYNUCLEOTIDES AND METHODS OF PREPARING SAME

The invention disclosed and/or claimed in this application was made in the course of work carried out under grants from the Public Health Service, National Institute of General Medical Research Grant No. P50-GM20124; Public Health Service, National Cancer Institute Training Grant No. T32-CA09159; and Public Health Service, National Institute of General Medical Science Grant No. T32-GM07499. The Government has certain rights in the invention.

This is continuation of application Ser. No. 07/130,002, filed Dec. 8, 1987, now abandoned, which is in turn a division of application Ser. No. 06/496,915, filed May 23, 1983, issued Dec. 8, 1987 as U.S. Pat. No. 4,711,955. Application Ser. No. 06/496,915 is in turn a continuation of application Ser. No. 06/255,223, filed Apr. 17, 1981, now abandoned.

BACKGROUND OF THE INVENTION

Many procedures employed in biomedical research and recombinant DNA technology rely heavily on the use of nucleotide or polynucleotide derivatives radioactively labeled with isotopes of hydrogen (^{3}H), phosphorous (^{32}p), carbon (^{14}C), or iodine (^{125}I). Such radioactive compounds provide useful indicator probes that permit the user to detect, monitor, localize, or isolate nucleic acids and other molecules of scientific or clinical interest, even when present in only extremely small amounts. To date, radioactive materials have provided the most sensitive, and in many cases the only, means to perform many important experimental or analytical tests. There are, however, serious limitations and drawbacks associated with the use of radioactive compounds. First, since personnel who handle radioactive material can be exposed to potentially hazardous levels of radiation, elaborate safety precautions must be maintained during the preparation, utilization, and disposal of the radioisotopes. Secondly, radioactive nucleotides are extremely expensive to purchase and use, in large part due to the cost of equipment and manpower necessary to provide the appropriate safeguards, producer-/user health monitoring services, and waste-disposal programs. Thirdly, radioactive materials are often very unstable and have a limited shelf-life, which further increases usage costs. This instability results from radiolytic decomposition, due to the destructive effects associated with the decay of the radioisotope itself, and from the fact that many isotopes (e.g. ^{32}p and ^{125}I) have half-lives of only a few days.

It is known that haptens can combine with antibodies, but can initiate an immune response only if bound to a carrier. This property can be exploited in detection and identification testing.

It is also known that biotin and iminobiotin strongly interact with avidin, a 68,000 dalton glycoprotein from egg white. This interaction exhibits one of the tightest, non-covalent binding constants ($K_{diss} = 10^{-15}$) seen in nature. If avidin is coupled to potentially demonstrable indicator molecules, including fluorescent dyes, e.g. fluorescein or rhodamine, electron-dense reagents, e.g. ferritin, hemocyanin, or colloidal gold, or enzymes capable of depositing insoluble reaction products, e.g. peroxidase or alkaline phosphatase, the presence, location, or quantity of a biotin probe can be established. Although iminobiotin binds avidin less tightly than biotin, similar reactions can be used for its detection. Moreover, the reversibility of the iminobiotin-avidin interaction, by decreasing solution pH, offers significant advantages in certain applications.

The specificity and tenacity of the biotin-avidin complex has been used in recent years to develop methods for visually localizing specific proteins, lipids, or carbohydrates on or within cells (reviewed by E. A. Bayer and M. Wilchek in Methods of Biochemical Analysis, 26, 1, 1980). Chromosomal location of RNA has been determined by electron microscopy using a biotinized protein, cytochrome C, chemically crosslinked to RNA as a hybridization probe. The site of hybridization was visualized through the binding of avidin-ferritin or avidin-methacrylate spheres mediated by the avidin-biotin interaction. (J. E. Manning, N. D. Hershey, T. R. Broker, M. Pellegrini, H. K. Mitchell, and N. Davidson, Chromosoma, 53, 107, 1975; J. E. Manning, M. Pellegrini, and N. Davidson, Biochemistry, 61, 1364, 1977; T. R. Broker, L. M. Angerer, P. H. Yen, N. D. Hersey, and N. Davidson, Nucleic Acid Res., 5, 363, 1978; A. Sodja and N. Davidson, Nucleic Acid Res., 5, 383, 1978.) This approach to the detection of polynucleotide sequences, although successful in the specialized cases examined which were highly reiterated sequences, is not of general utility for analysis of polynucleotides present in single or low copy number.

Moreover, methods for attaching chemical moieties to pyrimidine and purine rings are known. Several years ago a simple and rapid acetoxymercuration reaction was developed for introducing covalently bound mercury atoms into the 5-position of the pyrimidine ring, the C-8 position of the purine ring or the C-7 position of a 7-deazapurine ring, both in nucleotides and polynucleotides. (R. M. K. Dale, D. C. Livingston and D. C. Ward, Proc. Natl. Acad. Sci. U.S.A., 70, 2238, 1973; R. M. K. Dale, E. Martin, D. C. Livingston and D. C. Ward, Biochemistry, 14, 2447, 1975.) It was also shown several years ago that organomercurial compounds would react with olefinic compounds in the presence of palladium catalysts to form carbon-carbon bonds (R. F. Heck, J. Am. Chem. Soc., 90, 5518, 1968; R. F. Heck, Ibid., 90, 5526, 1968; R. F. Heck, Ibid., 90, 5531, 1968; R. F. Heck, Ibid., 90, 5535, 1968; and R. F. Heck, J. Am. Chem. Soc. 91, 6707, 1969.) Bergstrom and associates (J. L. Ruth and D. E. Berstrom, J. Org. Chem., 43, 2870, 1978, and D. E. Bergstrom and M. K. Ogawa, J. Am. Chem. Soc., 100, 8106, 1978) and Bigge, et al. (C. F. Bigge, P. Kalantis, J. R. Deck and M. P. Mertes, J. Am. Chem. Soc., 102, 2033, 1980) have recently applied this reaction scheme in the synthesis of C-5 substituted pyrimidine nucleotide compounds.

Finally, it is known that antibodies specific for modified nucleotides can be prepared and used for isolating and characterizing specific constituents of the modified nucleotides. (T. W. Munns and M. K. Liszewski, Progress in Nucleic Acid Research and Molecular Biology, 24, 109, 1980.) However, none of the antibodies prepared to date against naturally occurring nucleotides have been shown to react with their nucleotide determinant when it exists in a double-stranded RNA or DNA duplex or when in DNA-RNA hybrid molecules.

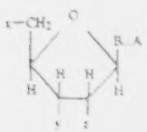
To circumvent the limitations of radioactively labeled probes or previously utilized chemical and biological probes, a series of novel nucleotide derivatives that contain biotin, iminobiotin, lipoic acid, and other determinants attached covalently to the pyrimidine or purine ring have been synthesized. These nucleotide deriva-

tives, as well as polynucleotides and coenzymes that contain them, will interact specifically and uniquely with proteins such as avidin or antibodies. The interaction between modified nucleotides and specific proteins can be utilized as an alternative to radioisotopes for the detection and localization of nucleic acid components in many of the procedures currently used in biomedical and recombinant-DNA technologies. Methods employing these modified nucleotide-protein interactions have detection capacities equal to or greater than procedures which utilize radioisotopes and they often can be performed more rapidly and with greater resolving power.

These new nucleotide derivatives can be prepared relatively inexpensively by chemical procedures which have been developed and standarized as discussed more fully hereinafter. More significantly, since neither the nucleotide probes of this invention nor the protein reagents employed with them are radioactive, the compounds can be prepared, utilized, and disposed of, without the elaborate safety procedures required for radioisotopic protocols. Moreover, these nucleotide derivatives are chemically stable and can be expected to have functional shelf-lives of several years or more. Finally, these compounds permit the development of safer, more economical, more rapid, and more reproducible research and diagnostic procedures.

SUMMARY OF THE INVENTION

Compounds having the structure

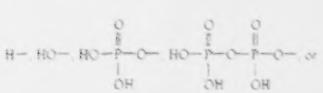


wherein B represents a purine, deazapurine, or pyrimidine moiety covalently bonded to the C^1-position of the sugar moiety, provided that when B is purine or 7-deazapurine, it is attached at the N^9-position of the purine or deazapurine, and when B is pyrimidine, it is attached at the N^1-position;

wherein A represents a moiety consisting of at least three carbon atoms which is capable of forming a detectable complex with a polypeptide when the compound is incorporated into a double-stranded ribonucleic acid, deoxyribonucleic acid duplex, or DNA-RNA hybrid;

wherein the dotted line represents a chemical linkage joining B and A, provided that if B is purine the linkage is attached to the 8-position of the purine, if B is 7-deazapurine, the linkage is attached to the 7-position of the deazapurine, and if B is pyrimidine, the linkage is attached to the 5-position of the pyrimidine; and

wherein each of x, y, and z represents



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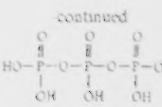
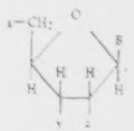


are widely useful as probes in biomedical research and recombinant DNA technology.

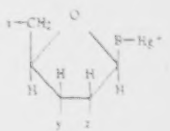
Particularly useful are compounds encompassed within this structure which additionally have one or more of the following characteristics: A is non-aromatic; A is at least C^5; the chemical linkage joining B and A includes an α-olefinic bond; A is biotin or iminobiotin; and B is a pyrimidine or 7-deazapurine.

These compounds may be prepared by a process which involves:

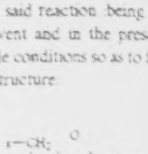
(a) reacting a compound having the structure:



with a mercuric salt in a suitable solvent under suitable conditions so as to form a mercurated compound having the structure:



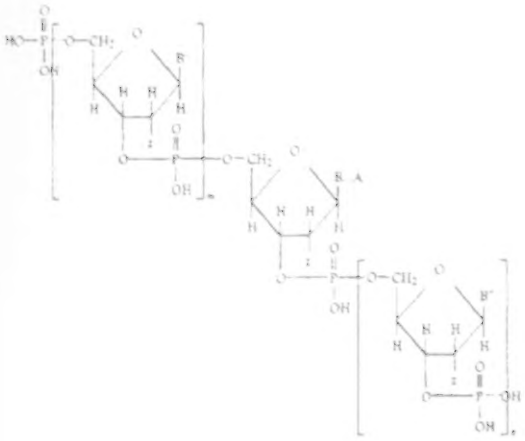
(b) reacting said mercurated compound with a chemical moiety reactive with the $-Hg^+$ portion of said mercurated compound and represented by the formula . . . N, said reaction being carried out in an aqueous solvent and in the presence of K_2PdCl_4 under suitable conditions so as to form a compound having the structure:



wherein N is a reactive terminal functional group or is A; and

(c) recovering said compound as said modified nucleotide when N is A, or when N is a reactive terminal group, reacting said compound with a compound having the structure M-A, wherein M represents a functional group reactive with N in an aqueous solvent under suitable conditions so as to form said modified nucleotide, which is then recovered.

This invention also provides compounds having the structure:



wherein each of B, B', and B'' represents a purine, 7-deazapurine, or pyrimidine moiety covalently bonded to the C^1-position of the sugar moiety, provided that whenever B, B', or B'' is purine or 7-deazapurine, it is attached at the N^9-position of the purine or 7-deazapurine, and whenever B, B', or B'' is pyrimidine, it is attached at the N^1-position;

wherein A represents a moiety consisting of at least three carbon atoms which is capable of forming a detectable complex with a polypeptide when the compound is incorporated into a double-stranded duplex formed with a complementary ribonucleic or deoxyribonucleic acid molecule

wherein the dotted line represents a chemical linkage joining B and A, provided that if B is purine the linkage is attached to the 8-position of the purine, if B is 7-deazapurine, the linkage is attached to the 7-position of the deazapurine, and if B is pyrimidine, the linkage is attached to the 5-position of the pyrimidine;

wherein z represents H— or HO—; and

wherein m and n represent integers from 0 up to about 100,000

These compounds can be prepared by enzymatic polymerization of a mixture of nucleotides which include the modified nucleotides of this invention. Alternatively, nucleotides present in oligo- or polynucleotides may be modified using chemical methods.

Nucleotides modified in accordance with the practices of this invention and oligo- and polynucleotides into which the modified nucleotides have been incorporated may be used as probes in biomedical research, clinical diagnosis, and recombinant DNA technology. These various utilities are based upon the ability of the molecules to form stable complexes with polypeptides which in turn can be detected, either by means of properties inherent in the polypeptide or by means of detectable moieties which are attached to, or which interact with, the polypeptide.

Some uses include detecting and identifying nucleic acid-containing etiological agents, e.g. bacteria and viruses; screening bacteria for antibiotic resistance; diagnosing genetic disorders, e.g. thalassemia and sickle cell anemia; chromosomal karyotyping; and identifying tumor cells.

DETAILED DESCRIPTION OF THE INVENTION

Several essential criteria must be satisfied in order for a modified nucleotide to be generally suitable as a substitute for a radioactively-labeled form of a naturally occurring nucleotide. First, the modified compound must contain a substituent or probe that is unique, i.e., not normally found associated with nucleotides or polynucleotides. Second, the probe must react specifically with chemical or biological reagents to provide a sensitive detection system. Third, the analogs must be relatively efficient substrates for commonly studied nucleic acid enzymes, since numerous practical applications require that the analog be enzymatically metabolized, e.g., the analogs must function as substrates for nucleic acid polymerases. For this purpose, probe moieties should not be placed on ring positions that sterically, or otherwise, interfere with the normal Watson-Crick hydrogen bonding potential of the bases. Otherwise, the substituents will yield compounds that are inactive as polymerase substrates. Substitution at ring positions that alter the normal "anti" nucleoside conformation also must be avoided since such conformational changes usually render nucleotide derivatives unacceptable as polymerase substrates. Normally, such considerations limit substitution positions to the 5-position of a pyrimidine and the 7-position of a purine or a 7-deazapurine.

Fourth, the detection system should be capable of interacting with probe substituents incorporated into both single-stranded and double-stranded polynucleotides in order to be compatible with nucleic acid hybridization methodologies. To satisfy this criterion, it is preferable that the probe moiety be attached to the purine or pyrimidine through a chemical linkage or "linker arm" so that it can readily interact with antibodies, other detector proteins, or chemical reagents.

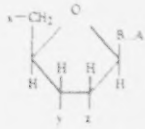
Fifth, the physical and biochemical properties of polynucleotides containing small numbers of probe substituents should not be significantly altered so that current procedures using radioactive hybridization

probes need not be extensively modified. This criterion must be satisfied whether the probe is introduced by enzymatic or direct chemical means.

Finally, the linkage that attaches the probe moiety should withstand all experimental conditions to which normal nucleotides and polynucleotides are routinely subjected, e.g., extended hybridization times at elevated temperatures, phenol and organic solvent extraction, electrophoresis, etc.

All of these criteria are satisfied by the modified nucleotides described herein.

These modified nucleotides have the structure

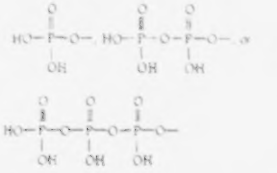


wherein B represents a purine, 7-deazapurine, or pyrimidine moiety covalently bonded to the $C^{1'}$-position of the sugar moiety, provided that when B is purine or 7-deazapurine, it is attached at the N^{9}-position of the purine or 7-deazapurine, and when B is pyrimidine, it is attached at the N^{1}-position;

wherein A represents a moiety consisting of at least three carbon atoms which is capable of forming a detectable complex with a polypeptide when the compound is incorporated into a double-stranded ribonucleic acid, deoxyribonucleic acid duplex, or DNA-RNA hybrid;

wherein the dotted line represents a linkage group joining B and A, provided that if B is purine the linkage is attached to the 8-position of the purine, if B is 7-deazapurine, the linkage is attached to the 7-position of the deazapurine, and if B is pyrimidine, the linkage is attached to the 5-position of the pyrimidine; and

wherein each of x, y and z represents



These compounds are widely useful as probes in biomedical research and recombinant DNA technology.

Although in principal all compounds encompassed within this structural formula may be prepared and used in accordance with the practices of this invention, certain of the compounds are more readily prepared or used or both, and therefore are presently preferred.

Thus, although purines, pyrimidines and 7-deazapurines are in principal useful, pyrimidines and 7-deazapurines are preferred since purine substitution at the 8-position tends to render the nucleotides ineffective as polymerase substrates. Thus, although modified purines are useful in certain respects, they are not as generally useful as pyrimidines and 7-deazapurines. Moreover, pyrimidines and 7-deazapurines useful in this invention must not be naturally substituted at the 5-or 7- positions, respectively. As a result, certain bases such as thymine, 5-methylcytosine, and 5-hydroxymethylcytosine are not useful. Presently preferred bases are cytosine, uracil, deazaadenine and deazaguanine.

A may be any moiety which has at least three carbon atoms and is capable of forming a detectable complex with a polypeptide when the modified nucleotide is incorporated into a double-stranded duplex containing either deoxyribonucleic or ribonucleic acid.

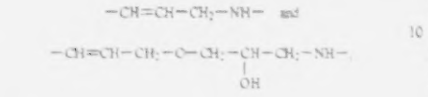
A therefore may be any ligand which possesses these properties, including haptens which are only immunogenic when attached to a suitable carrier, but are capable of interacting with appropriate antibodies to produce complexes. Examples of moieties which are useful include:

Of these the preferred A moieties are biotin and iminobiotin.

Moreover, since aromatic moieties tend to intercalate into a base-paired helical structure, it is preferred that the moiety A be nonaromatic. Also, since smaller moieties may not permit sufficient molecular interaction with polypeptides, it is preferred that A be at least C_5 so that sufficient interaction can occur to permit formation of stable complexes. Biotin and iminobiotin satisfy both of these criteria.

The linkage or group joining moiety A to base B may include any of the well known bonds including carbon-carbon single bonds, carbon-carbon double bonds, carbon-nitrogen single bonds, or carbon-oxygen single bonds. However, it is generally preferred that the chemical linkage include an olefinic bond at the α-position relative to B. The presence of such an α-olefinic bond serves to hold the moiety A away from the base when the base is paired with another in the well known double-helix configuration. This permits interaction with polypeptide to occur more readily, thereby facilitating complex formation. Moreover, single bonds with greater rotational freedom may not always hold the moiety sufficiently apart from the helix to permit recognition by and complex formation with polypeptide.

It is even more preferred that the chemical linkage group be derived from a primary amine, and have the structure —CH_2—NH—, since such linkages are easily formed utilizing any of the well known amine modification reactions. Examples of preferred linkages derived from allylamine and allyl-(3-amino-2-hydroxy-1-propyl) ether groups have the formulae



—CH=CH—CH_2—NH— and

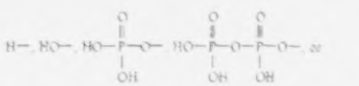
—CH=CH—CH_2—O—CH_2—CH(OH)—CH_2—NH—,

respectively.

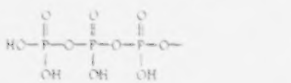
Although these linkages are preferred, others can be used, including particularly olefin linkage arms with other modifiable functionalities such as thiol, carboxylic acid, and epoxide functionalities.

The linkage groups are attached at specific positions, namely, the 5-position of a pyrimidine, the 8-position of a purine, or the 7-position of a deazapurine. As indicated previously, substitution at the 8-position of a purine does not produce a modified nucleotide which is useful in all the methods discussed herein. It may be that the 7-position of a purine, which is occupied by a nitrogen atom, could be the point of linkage attachment. However, the chemical substitution methods employed to date and discussed herein are not suitable for this purpose.

The letters x, y, and z represent groups attached to the 5', 3', and 2' positions of the sugar moiety. They may be any of



H—, HO—, HO—P(=O)(OH)—O—, HO—P(=O)(OH)—O—P(=O)(OH)—O—, or



Although conceivable, it is unlikely that all of x, y, and z will simultaneously be the same. More likely at least one of x, y, and z will be a phosphate-containing group, either mono-, di-, or tri-phosphate and at least one will be HO— or H—. As will be readily appreciated, the most likely identity of z will be HO— or H— indicating ribonucleotide or deoxyribonucleotide, respectively. Examples of such nucleotides include 5'-ribonucleoside monophosphates, 5'-ribonucleoside diphosphates, 5'-ribonucleoside triphosphates, 5'-deoxyribonucleoside monophosphates, 5'-deoxyribonucleoside diphosphates, 5'-deoxyribonucleoside triphosphates, 5'p-ribonucleoside-3'p, and 5'p-deoxyribonucleoside-3'p. More specific examples include modified nucleotides of this type in which A is biotin or iminobiotin, the chemical linkage is

—CH=CH—CH_2—NH— or

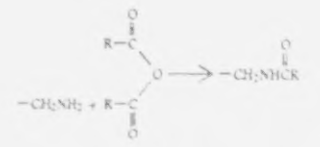
—CH=CH—CH_2—O—CH_2—CH(OH)—CH_2—NH—,

and B is uracil or cytosine.

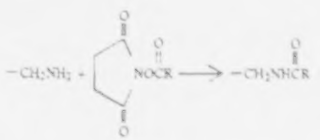
The general synthetic approach adopted for introducing the linker arm and probe moiety onto the base is discussed hereinabove. (See especially, J. L. Ruth and D. E. Bergstrom, Org. Chem., 43, 2870, 1978; D. E. Bergstrom and M. K. Ogawa, J. Amer. Chem. Soc. 100, 8106, 1978; and C. F. Bigge, P. Kalaritis, J. R. Deck and M. P. Mertes, J. Amer. Chem. Soc. 102, 2033, 1980.) However, the olefin substituents employed herein have not been used previously. To facilitate attachment of probe moiety A, it has been found particularly desirable to employ olefins with primary amine functional groups, such as allylamine [AA] or allyl-(3-amino-2-hydroxy-1-propyl) ether [NAGE], which permit probe attachment by standard amine modification reactions, such as,

$-CH_2NH_2 + R-C(=NH_2^+)-OR \longrightarrow -CH_2NHCR$

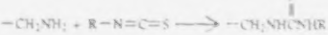
Imidate



Anhydride



NHS-ester (N-hydroxysuccinimide)



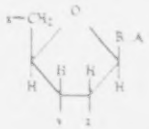
$-CH_2NH_2 + R-N=C=S \longrightarrow -CH_2NHCNHR$

Isothiocyanate

Epoxide

Because of ease of preparation it has been found preferable to use NHS-esters for probe addition. However, olefin linker arms with other modifiable functional groups, such as thiols, carboxylic acids, epoxides, and the like, can also be employed. Furthermore, both linker arm and probe can be added in a single-step if deemed desirable.

Specifically, modified nucleotides having the structure



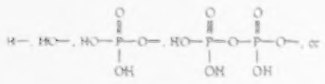
wherein B represents a purine, 7-deazapurine, or pyrimidine moiety covalently bonded to the C1'-posi-

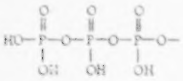
tion of the sugar moiety, provided that when B is purine or 7-deazapurine, it is attached at the N^9-position of the purine or deazapurine, and when B is pyrimidine, it is attached at the N^1-position;

wherein A represents a moiety consisting of at least three carbon atoms which is capable of forming a detectable complex with a polypeptide when the compound is incorporated into a double-stranded ribonucleic acid, deoxyribonucleic acid duplex, DNA-RNA hybrid;

wherein the dotted line represents a chemical linkage joining B and A, provided that if B is purine, the linkage is attached to the 8-position of the purine, if 7-deazapurine, the linkage is attached to the 7-position of the deazapurine, and if B is pyrimidine, the linkage is attached to the 5-position of the pyrimidine; and

wherein each of x, y, and z represents

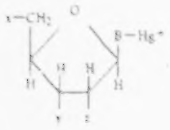




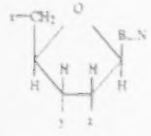
can be prepared by:

(a) reacting a compound having the structure:

with a mercuric salt in a suitable solvent under suitable conditions so as to form a mercurated compound having the structure:



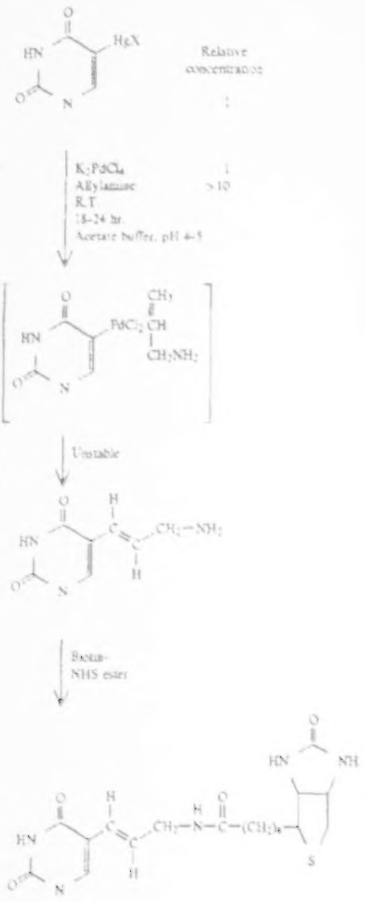
(b) reacting said mercurated compound with a chemical moiety reactive with the $-Hg^+$ portion, of said mercurated compound and represented by the formula . . . N, said reaction being carried out in an aqueous solvent and in the presence of K_2PdCl_4 under suitable conditions so as to form a compound having the structure:



wherein N is a reactive terminal functional group or is A, and

(c) recovering said compound as said modified nucleotide when N is A, or when N is a reactive terminal group, reacting said compound with a compound having the structure M-A, wherein M represents a functional group reactive with N in an aqueous solvent under suitable conditions, so as to form said modified nucleotide, which is then recovered.

The following schema is illustrative:



Although the reactions can be carried out at hydrogen ion concentrations as low as pH 1, or as high as pH 14, it is preferred to operate in the range from about 4 to 8. This is especially true when dealing with unstable compounds such as nucleoside polyphosphates, polynucleotides, and nucleotide coenzymes which are hydrolyzed at pH's outside this range. Similarly, it is preferred to operate at a temperature in the range from about 20° C. to 30° C. to avoid possible decomposition of labile organic substrates. However, the reactions can be carried out at temperatures from about 5° C. to 100° C. As

is usual with chemical reactions, higher temperatures promote the reaction rate and lower temperatures retard it. Thus, in the temperature range from 5° C. to 100° C., the optimum reaction time may vary from about 10 minutes to 98 hours. In the preferred temperature range, reaction times normally vary from about 3 to 24 hours.

The preferred procedure for maintaining the pH in the desired range is through the use of buffers. A variety of buffers can be employed. These include, for example, sodium or potassium acetate, sodium or potassium citrate, potassium citrate-phosphate, tris-acetate and borate-sodium hydroxide buffers. The concentration of buffer, when employed, can vary over a wide range, up to about 2.0 molar.

While a particular advantage of the mercuration and palladium catalyzed addition reactions is that they can be carried out in water, small amounts of an organic solvent can be usefully included as a solubility aid. The organic solvents usually chosen are those which are miscible with water. These may be selected from ethers, alcohols, esters, ketones, amides, and the like such as methanol, ethanol, propanol, glycerin, dioxane, acetone, pyridine and dimethylformamide. However, since it has been observed that the presence of alcohols, such as methanol, often results in alkoxy-addition across the olefin double bond, any organic solvent used as a solubility aid should be chosen carefully. Introduction of alkoxy substituents to the α- or β-exocyclic carbon atoms often results in the production of compounds which are utilized much less efficiently as enzyme substrates.

Although various mercuric salts may be utilized, the presently preferred salt is mercuric acetate. Also, as indicated previously, the compounds may be prepared by first adding a linker arm and then the moiety A, or by adding a linker arm to which A is already attached. Thus, the chemical moiety represented by the formula . . . N may be any one of the numerous entities which ultimately result in production of the desired compounds.

Examples include

$$-CH=CH-CH_2-NH_2$$

$$-CH=CH-CH_2-O-CH_2-\underset{\displaystyle OH}{\underset{|}{CH}}-CH_2-NH_2,$$

$$-CH=CH-CH_2-NH\text{-biotin, and}$$

$$-CH=CH_2-CH_2-O-CH_2-\underset{\displaystyle OH}{\underset{|}{CH}}-CH_2-NH\text{-aminobiotin}$$

The amounts of the reactants employed in these reactions may vary widely. However, in general the amounts of unmercurated compound, mercurated compound, and palladium-containing compound will be substantially stoichiometric whereas the mercuric salt and compound . . . N will be present in molar excess, e.g. 5-20 moles of . . . N or of mercuric salt per mole of mercurated compound or unmercurated compound, respectively. In practice, amounts will vary depending upon variations in reaction conditions and the precise identity of the reactants.

Having the biotin probe directly attached to nucleotide derivatives that are capable of functioning as enzyme substrates offers considerable versatility, both in the experimental protocols that can be performed and in the detection methods (microscopic and non-microscopic) that can be utilized for analysis. For example, biotin nucleotides can be introduced into polynucleotides which are in the process of being synthesized by cells or crude cell extracts, thus making it possible to detect and/or isolate nascent (growing) polynucleotide chains. Such a procedure is impossible to do by any direct chemical modification method. Furthermore, enzymes can be used as reagents for introducing probes such as biotin into highly selective or site-specific locations in polynucleotides; the chemical synthesis of similar probe-modified products would be extremely difficult to achieve at best.

The synthesis of nucleotides containing biotin or iminobiotin was achieved as detailed in the examples set forth hereinafter. Pyrimidine nucleoside triphosphates containing either of these probes attached to the C-5 carbon atom were good to excellent substrates for a wide variety of purified nucleic acid polymerases of both prokaryotic and eukaryotic origin. These include DNA polymerase I of *E. coli*, bacteriophage T4 DNA polymerase, DNA polymerases α and β from murine (A-9) and human (HeLa) cells, and the DNA polymerase of Herpes simplex virus. Confirming data were obtained with *E. coli* DNA polymerase I using either the nicktranslation condition of Rigby, et al. (P. W. J. Rigby, M. Dieckmann, C. Rhodes and P. Berg, J. Mol. Biol. 113, 237, 1977) or the gap-filling reaction described by Bourguignon et al. (G. J. Bourguignon, P. J. Tattersall and D. C. Ward, J. Virol. 20, 290, 1976). Bio-dUTP has also been found to function as a polymerase substrate both in CHO cells, permeabilized by treatment with lysolecithin according to the method of Miller, et al. (M. R. Miller, J. C. Casteliot, Jr and A. B. Pardee, Exp. Cell Res. 120, 421, 1979) and in a nuclear replication system prepared from Herpes simplex infected BHK cells. Although biotinyl ribonucleoside triphosphates were found to function as substrates for the RNA polymerases of *E. coli* and bacteriophage T7, they are not utilized as efficiently as their deoxyribonucleotide triphosphate counterparts. Indeed, they are incorporated poorly, if at all, by the eukaryotic RNA polymerases examined (HeLa cell RNA polymerase III, calf thymus RNA polymerase II and mouse cell RNA polymerase II). While this limited range of substrate function does restrict the utility in some in vivo or in vitro transcription studies, biotinlabeled RNA probes can be prepared enzymatically from DNA templates using *E. coli* or T7 RNA polymerases or by 3' endlabeling methods using RNA ligase with compounds such as biotinyl-pCp. The AA- and NAGE-derivatives of UTP are, however, substrates for the eukaryotic RNA polymerases mentioned above. With the availability of antibodies to these analogs, the isolation of nascent transcripts by immunological or affinity procedures should be feasible.

The enzymatic polymerization of nucleotides containing biotin or iminobiotin substituents was not monitored directly, since neither of these probes were radiolabeled. However, two lines of experimental evidence clearly show that the biotinyl-nucleotides were incorporated. The first is that polynucleotides synthesized in the presence of biotinnucleotides are selectively retained when chromatographed over avidin or streptavidin affinity columns. (Tables I and II) For example, whereas normal DNA, nick translated with ^{32}P-dAMP, is quantitatively eluted upon the addition of 0.5M NaCl,

the vast majority of biotinyl-DNA or iminobiotinylDNA remains bound to the resin even after extensive washing with high salt, urea, guanidine-HCl, formamide or 50 mM NaOH. The small fraction of the radiolabel eluted by these washing conditions is not retained when applied to the resin a second time, suggesting that radioactivity is associated with DNA fragments which are free of biotin substitution. The second line of evidence is that only biotin-labeled polynucleotides are immunoprecipitated when treated with purified anti-biotin IgG followed by formalin-fixed *Staphylococcus aureus*. (Table III) It is-clear from the data in these tables that extremely small amounts of biotin can be detected by this method. These results also show that the biotin molecule can be recognized by avidin, streptavidin or specific antibodies while the DNA is still in its native, double-stranded form, a condition that is absolutely essential if the antibody-binding or avidin-affinity approaches are to be useful in probe detection employing hybridization techniques.

TABLE I

SELECTIVE RETENTION OF BIOTINIZED DNA ON AVIDIN-SEPHAROSE

Eluent		% DNA Retained on Resin Bio-DNA (1%)	T-DNA
Load -	3 × 105 cpm 10 mM Tris 7.5 + 0.2M NaCl	100	100%
(1)	0.5M NaCl	100	0.1
(2)	1.0M NaCl	99.7	<0.01
(3)	8M Urea	100	<0.01
(4)	6M guanidine-HCl	95.2	<0.01
(5)	99% formamide	94.7	<0.01
(6)	2 mM Biotin	87.6	<0.01
(7)	50 mM NaOH	89.5	<0.01

TABLE II

Affinity Chromatography of Iminobiotin-dUTP and Iminobiotinized - DNA on Streptavidin-Sepharose

Eluent		% Retained on SA-Sepharose T-DNA	^{3}H-IB-dUTP	IB-DNA
Load -	10 mM Tris HCl, 8.3 50 mM NaCl	8.7	100	99.7
(1)	0.1M NaCl	<0.1	100	99.7
(2)	1.0M NaCl	<0.01	100	99.4
(3)	8M Urea	<0.01	97.5	98.5
(4)	6M guanidine-HCl	<0.01	97.0	97.0
(5)	50 mM NH_4-acetate, pH 4.0	<0.01	<0.01	96.5
(6)	50 mM NH_4-acetate, pH 4.0 2 mM biotin	<0.01	<0.01	<0.01

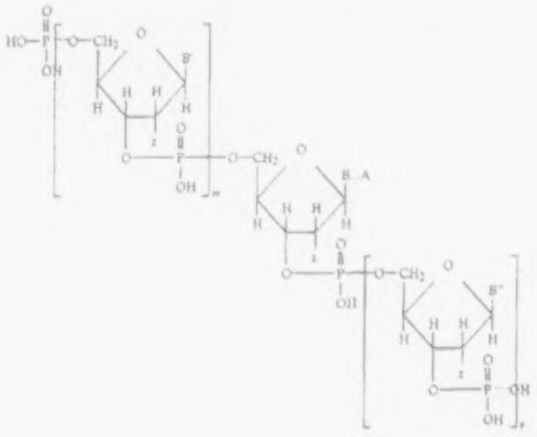
TABLE III

SELECTIVE IMMUNOPRECIPITATION OF BIO-DNA WITH ANTI-BIOTIN IgG and *STAPH AUREUS*

DNA*	Antibody	CPM in Immuno ppt.	CPM in Supernatant
T-DNA	–	70	4867
T-DNA	Anti-Bio IgG	87	5197
T-DNA	Non-immune IgG	55	5107
Bio-DNA	–	53	3836
Bio-DNA	Anti-Bio IgG	3347	736
Bio-DNA	Non-immune IgG	60	3900

*N.T. pBR-322 DNA, ^{32}P-labeled, 1% Biotin substitution.
Specific activity 2 × 10^7 cpm/μg.
Biotin detection 0.001-0.01 picoles.

Thus, it is possible to prepare novel compounds having the structure:



wherein each of B, B', and B'' represents a purine, deazapurine, or pyrimidine moiety covalently bonded to the C^1'-position of the sugar moiety, provided that whenever B, B', or B'' is purine or 7-deazapurine, it is attached at the N^9-position of the purine or deazapurine, and whenever B, B', or B'' is pyrimidine, it is attached at the N^1-position,

wherein A represents a moiety consisting of at least three carbon atoms which is capable of forming a detectable complex with a polypeptide when the compound is incorporated into a double-stranded duplex formed with a complementary ribonucleic or deoxyribonucleic acid molecule.

wherein the dotted line represents a linkage group joining B and A, provided that if B is purine, the linkage is attached to the 8-position of the purine, if B is 7-deazapurine, the linkage is attached to the 7-position of the deazapurine, and if B is pyrimidine, the linkage is attached to the 5-position of the pyrimidine;

wherein z represents H— or HO —; and

wherein m and n represent integers from 0 up to about 100,000.

Of course, it should be readily understood that in general m and n will not simultaneously be 0 since, in that event, the compound becomes merely a modified nucleotide as described previously. In general B' and B" will vary within the same oligo- or polynucleotide, being alternatively uracil, cytosine, thymine, guanine, adenine, or the like. Also, in general, the variation will correspond to the ordered sequence of nucleotides which codes for the synthesis of peptides according to the well known Genetic Code. However, it is intended that the structure shown also embrace polynucleotides such as poly C, poly U, poly r(A-U), and poly d(A-U) as well as calf thymus DNA, ribosomal RNA of *E. coli* or yeast, bacteriophage RNA and DNA (R17, fd), animal viruses (SV40 DNA), chromosomal DNA, and the like, provided only that the polynucleotides be modified in accordance with this invention.

It is also to be understood that the structure embraces more than one modified nucleotide present in the oligomer or polymer, for example, from two to thirty modified nucleotides. The critical factor in this regard is that the number of modifications not be so great that the polynucleotide is rendered ineffective for the intended use.

Finally, it should be understood that modified oligo- and polynucleotides can be joined to form larger entities having the same structure so long as terminal groups are rendered compatible or reactive. These compounds can be made by enzymatic polymerization of appropriate nucleotides, especially nucleotide triphosphates in the presence of a nucleic acid template which directs synthesis under suitable conditions. Such conditions can vary widely depending upon the enzyme employed, amounts of nucleotides present, and other variables. Illustrative enzymes include DNA polymerase I of *E. coli*, bacteriophage T4 DNA polymerase, DNA polymerases α and β from murine and human (HeLa) cells, DNA polymerase from Herpes simplex virus, RNA polymerase of *E. coli*, RNA polymerase of bacteriophage T7, eukaryotic RNA polymerase including HeLa cell RNA polymerase III, calf thymus RNA polymerase II, and mouse cell RNA polymerase II.

Also, the compounds can be prepared by terminal addition to oligo- or polynucleotides to produce compounds in which m or n is 0 depending upon whether the addition is at the 5' or 3' position. Moreover, the compounds such as pCp or pUp in which the base is biotinized can be added to existing molecules employing the enzyme RNA ligase.

Modified oligo- and polynucleotides can also be prepared by chemical modification of existing oligo- or polynucleotides using the approach described previously for modification of individual nucleotides.

The various modified nucleotides, oligonucleotides, and polynucleotides of this invention may be detected by contacting the compounds with polypeptides which are capable of forming complexes therewith under suitable conditions so as to form the complexes, provided that the polypeptides include one or more moieties which can be detected when the complex or complexes is or are formed, generally by means of conventional detection techniques.

One polypeptide detector for the biotinyl-type probe is avidin. The avidin-biotin interaction exhibits one of the tightest non-covalent binding constants ($K_{diss}=10^{-15}$) seen in nature. If avidin is coupled to potentially demonstrable indicator molecules, e.g., fluorescent dyes (fluoroscein, rhodamine), electron-dense reagents (ferritin, hemocyanin, colloidal gold), or enzymes capable of depositing insoluble reaction products (peroxidase, alkaline phosphatase) the presence, location and/or quantity of the biotin probe can be established.

Avidin has, unfortunately, one property that makes it less desirable as a biotin-indicator protein when used in conjunction with nucleic acids or chromatin material. It has been reported (M. H. Heggeness, Stain Technol., 52, 165, 1977; M. H. Heggeness and J. F. Ash, J. Cell. Biol., 73, 783, 1977; E. A. Bayer and M. Wilchek, Methods of Biochemical Analysis 26, 1, 1980) that avidin binds tightly to condensed chromatin or to subcellular fractions that contain large amounts of nucleic acid in a manner which is independent of its biotin-binding property. Since avidin is a basic glycoprotein with a pI of 10.5, its histone-like character or its carbohydrate moieties are most likely responsible for these observed non-specific interactions.

A preferred probe for biotin-containing nucleotides and derivatives is streptavidin, an avidin-like protein synthesized by the soil organism *Streptomyces avidinii*. Its preparation and purification is described in Hoffman, et al., Proc. Natl. Acad. Sci., 77, 4666 (1980). Streptavidin has a much lower pI (5.0), is non-glycosylated, and shows much lower non-specific binding to DNA than avidin, and therefore offers potential advantages in applications involving nucleic acid detection methodology.

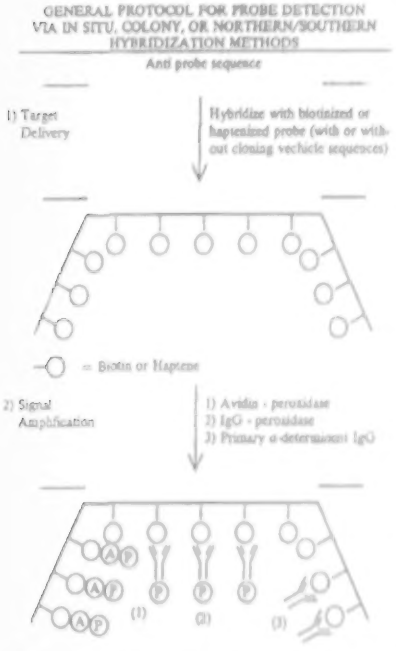
A most preferred protein for biotin-like probe detection is monospecific rabbit IgG, antibiotin immunoglobulin. This compound was prepared by immunizing rabbits with bovine serum albumin conjugated biotin as described previously (M. Berger, Methods in Enzymology, 62, 319 [1979]) and purified by affinity chromatography. Although the association constant of immunoglobulin-haptens have values of K_{assn} (10^6 to 10^{10}) which are considerably lower than for avidin-biotin complexes, they are substantially equivalent to those observed with the avidin-iminobiotin complex. Furthermore, the anti-biotin antibodies have proven extremely useful in detecting specific polynucleotide sequences on chromosomes by in situ hybridization since little, if any, non-specific binding of the antibody to chromatin material occurs.

The modified polynucleotides of this invention are capable of denaturation and renaturation under conditions compatible with their use as hybridization probes. An analysis of the thermal denaturation profiles and hybridization properties of several biotin-substituted DNA and RNA polymers clearly indicates this. For example, pBR 322 DNA or λ DNA, nick translated to introduce approximately 10–100 biotin residues per kilobase, have Tm values essentially identical to that of the control, biotin-free DNAs. Furthermore, ^{32}P-labeled, biotin-substituted, pBR 322 DNA, exhibited the same degree of specificity and autoradiographic signal intensity as control, thymidine-containing DNA, when

used as a hybridization probe for detecting bacterial colonies containing the plasmid.

In DNA duplexes, such as MVM RF DNA, in which every thymidine residue in one strand (1250 in toto per 5 Kb) is replaced by a biotinyl-nucleotide, the Tm is only 5° C. less than that of the unsubstituted control. Although the Tm of poly d(A-bioU) in which each base pair contains a bio-dUMP residue is 15° C. lower than the poly d(A-T) control, the degree of cooperativity and the extent of hyperchromicity observed both during denaturation and renaturation were the same for the two polymers. A parallel analysis of RNA duplexes and DNA/RNA hybrids indicates that their Tm's also decrease as the biotin-content of the polymer increases. However, it is clear that a substantial number of biotin-molecules can be introduced without significantly altering the hybridization characteristics of the polymers.

These results strongly suggested that biotin-substituted polynucleotides could be used as probes for detecting and/or localizing specific polynucleotide sequences in chromosomes, fixed cells, or tissue sections. The general protocol for detecting the biotin-substituted probe is schematically illustrated as follows:


GENERAL PROTOCOL FOR PROBE DETECTION VIA IN SITU, COLONY, OR NORTHERN/SOUTHERN HYBRIDIZATION METHODS
Anti probe sequence
1) Target Delivery
Hybridize with biotinized or haptenized probe (with or without cloning vechicle sequences)
= Biotin or Hapten
2) Signal Amplification
1) Avidin - peroxidase
2) IgG - peroxidase
3) Primary a-determinant IgG
(1)
(2)
(3)
3) Detection: 1) Insoluble peroxidase products. DAB
2) Antibody sandwiching techniques

This general scheme illustrates only procedures used for gene mapping (cytogenetics), and recombinant DNA-technologies. However, it can be equally well applied to the detection of nucleic acid sequences of bacterial, viral, fungal or parasite origin in clinical samples and this forms the basis of a powerful new approach to clinical diagnostics which does not rely on the use of radioisotopes.

Immunological and histochemical methods for the detection of biotin have shown that the basic approach is useable for a rapid method of gene mapping in situ hybridization and non-radioactive procedures for detecting specific nucleic acid sequences by blotting hybridization methods. Use may be made of this technology in development of new clinical diagnostic procedures.

Using this approach, it is possible to determine the presence of a specific deoxyribonucleic or ribonucleic acid molecule, particularly such a molecule derived from a living organism, e.g. bacteria, fungus, virus, yeast, or mammal. This in turn permits diagnosis of nucleic acid-containing etiological agents in a patient or other subject.

Moreover, it provides a method for screening bacteria to determine antibiotic resistance. Thus, for example, penicillin resistance in *Streptococcus pyogenes* or *Neisseria meningitidis*; tetracycline resistance in *Staphylococcus aureus, Candida albicans, Pseudomonas aeruginosa, Streptococcus pyogenes*, or *Neisseria gonorrhoeae*; and aminoglycoside resistance in *Mycobacterium tuberculosis* can be determined.

In these methods a polynucleotide is prepared which is complementary to the nucleic acid sequence which characterizes the organism or its antibiotic resistance and which additionally includes one or more modified nucleotides according to this invention. This polynucleotide is hybridized with nucleic acid obtained from the organism under scrutiny. Failure to hybridize indicates absence of the organism or of the resistance characteristic. Hybridized nucleic acid duplexes are then identified by forming a complex between the duplex and a suitable polypeptide which carries a detectable moiety, and detecting the presence of the complex using an appropriate detection technique. Positive detection indicates that the complex, the duplex and therefore the nucleic acid sequence of interest are present.

This approach can be extended to the diagnosis of genetic disorders, such as thalassemia and sickle cell anemia. The deoxyribonucleotide acid gene sequence whose presence or absence (in the case of thalassemia) is associated with the disorder can be detected following hybridization with a polynucleotide probe according to this invention based upon complex formation with a suitable detectable polypeptide.

The mapping of genes or their transcripts to specific loci on chromosomes has been a tedious and time-consuming occupation, involing mainly techniques of cell-fusion and somatic cell genetics. Although in situ hybridization has been employed successfully for mapping single-copy gene sequences in species that undergo chromosomes polytenization, such as Drosophila, detection of unique sequence genes in most higher eukaryotic chromosomes has been extremely difficult, if not impossible, using standard hybrization methods. The necessity for polynucleotide probes of very high specific radioactivity to facilitate autoradiographic localization of the hybridization site also results in rapid radio decomposition of the probe and a concomitant increase in the background noise of silver grain deposition. The use of hybridization probes with low to moderate specific radio activities requires exposure times of many days or weeks, even to detect multicopy sequences, such as ribosomal RNA genes or satellite DNA. Since recombinant DNA technology has made feasible the molecular cloning of virtually every single-copy sequence found in eukaryotic cells, it would be extremely beneficial to have a rapid and sensitive method

for mapping the chromosomal origin of such cloned genomic fragments.

Modified nucleotides may be used in a method of gene mapping by in situ hybridization which circumvents the use of radioisotopes. This procedure takes advantage of a thymidine analogue containing biotin that can be incorporated enzymatically into DNA probes by nick translation. After hybridization in situ the biotin molecules serve as antigens for affinity purified rabbit anti-biotin antibodies. Immunofluorescent antibody sandwiches made with fluorescein-labeled goat anti-rabbit IgG allow for rapid and specific cytogenetic localization of cloned gene sequences as green-yellow bands. This method offers four major advantages over conventional autoradiographic methods of in situ gene localization; less background noise, an increase in resolving power between bands, a decrease in the time required to determine the site of probe hybridization; and chemically stable hybridization probes. This method has been applied successfully to the localization of reiterated and unique DNA sequences in the polytene chromosome of *Drosophila melanogaster* and satellite DNA on mouse metaphase chromosomes.

Thus it has been found that polytene chromosomes could be used as a test system for establishing the efficacy of probes using the modified nucleotides according to the instant invention as detected by indirect immunofluorescence for in situ gene mapping. The probes included a variety of cloned Drosophila sequences obtained from Otto Schmidt and Dieter Söll, such as tRNA genes cloned in plasmid vectors with inserts of sizes ranging from about 5 to about 22 kilobases. Many of these clones have already been assigned to specific bands on the Drosophila chromosome map by conventional in situ hybridization methods employing radioisotopes.

DNA probes were nick translated in the presence of Bio-dUTP. Occasionally ^{3}H dATP and/or ^{3}H dCTP was included in the nick translation reaction mixture. This allowed both autoradiographic and immunofluorescent localization of a sequence on a single chromosome spread. In situ hybridization was performed as described in M. L. Pardue, and J. G. Gall, Methods in Cell Biol., 10, 1 (1975). After the final 2×SSC wash to remove unhybridized probe, the slides were rinsed with PBS (phosphate buffered saline) and incubated at 37° C. with 2.5 μg/ml Rabbit anti-biotin in PBS and 10 mg/ml BSA for 2-16 hours. This was followed by incubation of the slides with FITC labeled Goat anti-Rabbit IgG (Miles Laboratories, diluted 1:100 in PBS and 10 mg/ml BSA) for one-four hours. Evans Blue was often required as a red counterstain to set the chromosomes with fluorescent illumination.

When plasmids pBR 17D and pPW 532 containing 5 Kb and 22 Kb inserts, respectively, were hybridized by this method, it was found that the pattern of hybridization is reproducible from spread to spread and is observed unambiguously on greater than 90% of the chromosome spreads on a given slide.

The cloned transposable element pAC 104 is known to map at many sites along the Drosophila genome. Comparison of the autoradiograph and the fluorescent picture obtained by in situ hybridization of this probe illustrates a major advantage of this method, i.e., that where diffuse regions of silver grains appear on an autoradiograph, doublets or a series of bands are discernible by immunofluorescent labeling.

The other immediately obvious advantage of this method is the tremendous decrease in time required for gene assignments to be made by indirect immunofluorescence. An assignment of a DNA fragment to a specific band can be made within six hours of hybridization. This is in comparison to days or weeks required for autoradiographic exposure methods. This factor, in combination with increased resolution, makes the use of modified nucleotides detected by indirect immunofluorescence immediately preferable to more classical methods.

It has been shown that this immunological method also works with mammalian chromosomes wherein satellite DNA has been mapped to the centromeric regions of mouse metaphase chromosomes. The result provides a basic foundation for the development of a simple gene mapping procedure for single copy (unique) sequences in chromosomes from human and other mammals. Such a procedure should greatly facilitate our understanding of the genetic organization of the chromosome and make clinical cytogenetic diagnosis much more rapid and practical.

While a single-step "antibody sandwich" method in which the chromosome spread is challenged, post-hybridization, with rabbit anti-biotin IgG may succeed, this protocol may not generate sufficient fluorescence for unambiguous gene assignments. However, a much stronger fluorometric signal can be achieved by using the "haptene-antibody sandwich technique" described by Lamm, et al, (1972); Wofsy, et al., (1974). In this procedure the primary antibody, in our case monospecific, rabbit anti-biotin IgG, is chemically modified with a haptenization reagent, such as 2,4-dinitrofluorobenzene, preferably while the immunoglobulin is bound to an antigen affinity column (biotin-Sepharose TM). As many as 15-20 haptene (DNP) groups can be coupled to the primary antibody without decreasing its antigen binding affinity or specificity (Wallace and Wofsy, 1979). If the primary antibody treatment of the test sample is followed by an incubation with a fluorescently labeled anti-hapten IgG antibody, rather than a fluorescently labeled anti-IgG, a 5-7 fold increase in fluorescence signal can be achieved. Since one also has available monospecific guinea pig anti-DNP IgG, we can haptenize this secondary antibody with biotin and thus generate two anti-hapten IgG populations, DNP-labeled anti-biotin IgG and biotin-labeled anti-DNP IgG. If these can be used alternately to achieve several rounds of hapten -antibody sandwiching and then followed with fluorescently labeled protein A from *Staphylococcus aureus*, which binds specifically to IgG molecules from many mammalian species, it could result in an enormous amplification of the primary antibody signal with its concomitant utility.

The protein streptavidin from *Streptomyces avidini* is a potential alternative to anti-biotin IgG as a vehicle to specifically direct a coupled visualization system [e.g., fluorescent probes (above) or histochemical reagents (below)] to the site of the hybridized biotin-containing polynucleotide. One of streptavidin's advantages over anti-biotin IgG is that its affinity for biotin is $K_{assn} = 10^{15}$ whereas association constants for haptene-IgG interactions are 10^7 to 10^{10}. The fast reaction rate and extreme affinity mean that the time required to localize the biotinized probe will be minutes with streptavidin versus hours with immunologic reagents. Initial evaluations of a streptavidin detection system are currently in progress. Polytene chromosomes hybridized

with biotinized DNA probes will be incubated with streptavidin followed by a subsequent incubation with bovine serum albumin which has been doubly labeled with biotin and FITC (FITC, biotinyl-BSA). Since only one of the four streptavidin subunits is likely to be involved in binding at each biotinized DNA site, potentially one labeled BSA molecule can bind to each of the remaining three nonconjugated subunits of the streptavidin-biotinyl nucleotide complex. The fluorescence signal from this single streptavidin+FITC, biotinyl-BSA layer will be compared with a control using the basic "antibody sandwich method" described earlier.

If the "antibody sandwich" and streptavidin+FITC, biotinyl-BSA detection intensities are comparable, one can attempt to enhance the streptavidin+FITC, biotinyl-BSA system to single-copy copy sensitivity in a manner that parallels the multiple "haptene-antibody sandwich" approach. Since some of biotin groups on BSA will not be bound to the first layer of streptavidin, a second layer of streptavidin can be added until sufficient signal is obtained. For example, if in the second layer, only two streptavidin promoters bind to each first-layer BSA and each of these streptavidin protomers binds three FITC-biotinyl BSA molecules, then the second layer intensity will be twice as great as that from the first layer; for the third layer, with analogous binding stoichiometries, the fluorescent intensity will be 12-fold that of the first layer, so the total intensity will rapidly increase with successively added layers. There are plans to use a larger carrier protein such as thyroglobulin rather than BSA in order to maximize amounts of attached fluorescent and biotin probes. It may also be necessary to use a longer linker arm between the biotin probe and the carrier protein. A longer linker arm should sterically optimize the theoretical delivery of a biotinized fluorescent carrier molecule to each nonconjugated streptavidin subunit and maximize the number of streptavidin protomers in the subsequent layer which will bind to the biotinized fluorescent carrier. As before, appropriate controls will be done to insure that substitution of the carrier protein with fluorescent probes and biotin does not cause solubility and/or nonspecific binding problems.

The streptavidin-carrier protein delivery system has two significant advantages over the immunofluorescent approach in addition to its speed of delivery. First, only two protein components are needed to form the layers. Second, only the carrier protein needs to be modified and it is not necessary to maintain functional or even total structural integrity as long as the biotin groups are accessible to streptavidin.

An alternative to the fluorescence method for visualizing hybridized probes is to direct enzymes such as peroxidase, alkaline phosphatase of β-galactosidase to the hybridization site where enzymatic conversion of soluble substrates to insoluble colored precipitates permits light microscope visualization. The important advantage of this technique is that the histochemical methods are 10 to 100-fold more sensitive than fluorescence detection. In addition, the colored precipitates do not bleach with extensive light exposure thus avoiding one of the general disadvantages of fluorescent light microscopy. These enzymes can be coupled to the final antibody instead of fluorescent probes in the "haptene-antibody sandwich" technique using bifunctional reagents such as glutaraldehyde or in the case of peroxidase via oxidation of the peroxidase carbohydrate moieties to aldehydes and coupling of these residues with ϵ-amino groups of the desired protein. For the streptavidin-biotinized carrier protein method, an enzyme with biotinyl groups coupled to it could replace a fluorescently-biotinized carrier system. Alternately, the enzyme could be coupled via biotin to the last layer of streptavidin with amplification of streptavidin sites being built up in preceding layers using biotinized BSA or thyroglobulin. We will begin developing the necessary histochemical reagents and the appropriate substrate/insoluble product combinations for visualizing in situ hybridizations without background problems in the near future. The histochemical approaches to signal amplification should therefore be ready for trial in the summer of 1981.

Detecting and/or imaging very low levels of fluorescent light is possible using currently available image intensifiers or systems composed of lasers and photomultipliers. These methods permit the detection of light down to the level of individual photons. With suitable digital processing systems, images can be produced in which each point, i.e. each pixel, of the image is strictly proportional to the number of photons emitted by a point at the object. Using systems of this kind or flow systems in which the cells or parts of cells flow past a laser beam, one can obtain detection sensitivity increases for fluorescent material of factors between 100 and 1000 beyond that which can be detected by the eye. This increase is sufficient to detect the fluorescence of single copy genes.

In a preferred modification, analogs of dUTP and UTP that contain a biotin molecule covalently bound to the C-5 position of the pyrimidine ring through an allylamine linker arm have been synthesized. These biotinyl-nucleotides are efficient substrates for a variety of DNA and RNA polymerases in vitro. DNA containing low levels of biotin substitution (50 molecules or less/kilobase) has denaturation, reassociation and hybridization characteristics which are indistinguishable from that of unsubstituted control DNA.

Thus, this invention also provides a method of chromosomal karyotyping. In this method, modified polynucleotides are prepared which correspond to known genes and include modified nucleotides. These polynucleotides are hybridized with chromosomal deoxyribonucleic acid and the resulting duplexes contacted with appropriate polypeptides under suitable conditions to permit complex formation. The polypeptides include detectable moieties so that the location of the complexes can be determined and the location of specific genes thereby fixed.

Another embodiment of this invention involves detection of poly A-containing sequences using poly U in which some of the uracil bases have been modified to contain a probe. Yet another embodiment involves cyclic modified nucleotides in which two of x, y, and z are reacted to form the cyclic moiety

Such cyclic modified nucleotides may then be used to identify hormone receptor sites on cell surfaces which in turn can be used as a method of detecting cancer or tumor cells.

Finally, tumor cells can be diagnosed by preparing polynucleotides which are modified according to this invention and are complementary to the messenger ribonucleic acid synthesized from a deoxyribonucleic acid gene sequence associated with the production of polypeptides, such as α-fetal protein or carcinoembryonic antigen, the presence of which is diagnostic for specific tumor cells. Hybridization and detection of hybrid duplexes thus would provide a method for detecting the tumor cells.

The examples which follow are set forth to illustrate various aspects of the present invention but are not intended to limit in any way its scope as more particularly set forth in the claims.

EXAMPLE 1 and 2

Synthesis of biotinyl-UTP and biotinyl-dUTP

a) Preparation of Mercurated Nucleotides

UTP (570 mg, 1.0 mmole) or dUTP 554 mg, 1.0 mmole) was dissolved in 100 ml of 0.1M sodium acetate buffer pH 6.0, and mercuric acetate (1.59 gm, 5.0 mmoles) added. The solution was heated at 50° C. for 4 hours, then cooled on ice. Lithium chloride (392 mg, 9.0 mmoles) was added and the solution extracted six times with an equal volume of ethyl acetate to remove excess $HgCl_2$. The efficiency of the extraction process was monitored by estimating the mercuric ion concentration in the organic phase using 4,4'-bis(dimethylamino)-thiobenzophenone (A. N. Christoper, Analyst, 94, 392 (1969). The extent of nucleotide mercuration, determined spectrophotometrically following iodination of an aliquot of the aqueous solution as described by Dale et al. (R. M. K. Dale, D. C. Ward, D. C. Livingston, and E. Martin, Nucleic Acid Res. 2, 915 [1975]), was routinely between 90 and 100%. The nucleotide products in the aqueous layer, which often became cloudy during the ethyl acetate extraction, were precipitated by the addition of three volumes of icecold ethanol and collected by centrifugation. The precipitate was washed twice with cold absolute ethanol, once with ethyl ether, and then air dried. These thus prepared mercurated nucleotides were used for the synthesis of the allylamine-nucleotides without further purification.

b) Synthesis of allylamine-dUTP and allylamine-UTP

The mercurated nucleotides (of step a) were dissolved in 0.1M sodium acetate buffer at pH 5.0, and adjusted to a concentration of 20 mM (200 OD/ml at 267 nm). A fresh 2.0 M solution of allylamine acetate in aqueous acetic acid was prepared by slowly adding 1.5 ml of allylamine (13.3 mmoles) to 8.5 ml of ice-cold 4M acetic acid. Three ml (6.0 mmoles) of the neutralized allylamine stock was added to 25 ml (0.5 mmole) of nucleotide solution. One nucleotide equivalent of K_2PdCl_4, (163 mg, 0.5 mmole), dissolved in 4 ml of water, was then added to initiate the reaction. Upon addition of the palladium salt (Alfa-Ventron) the solution gradually turned black with metal (Hg and Pd) deposits appearing on the walls of the reaction vessel. After standing at room temperature for 18–24 hours, the reaction mixture was passed through a 0.45 mm membrane filter (nalgene) to remove most of the remaining metal precipitate. The yellow filtrate was diluted fivefold and applied to a 100 ml column of DEAE-Sephadex TM A-25 (Pharmacia). After washing with one column volume of 0.1M sodium acetate buffer at pH 5.0, the products were eluted using a one liter linear gradient (0.1-0.6M) of either sodium acetate at pH ~8.9, or triethylammonium bicarbonate (TEAB) at pH 7.5. The desired product was in the major UV-absorbing portion which eluted between 0.30 and 0.35M salt. Spectral analysis showed that this peak contained several products, final purification was achieved by reverse phase-HPLC chromatography on columns of Partisil-ODS2, using either 0.5M $NH_4H_2PO_4$ buffer at pH 3.3 (analytical separations), or 0.5M triethylammonium acetate at pH 4.3 (preparative separations) as eluents. The 5'-triphosphates of 5-(3-aminopropen-1-yl) uridine (the allylamine adduct to uridine) were the last portions to be eluted from the HPLC column and they were clearly resolved from three, as yet uncharacterized, contaminants. These nucleotides were characterized by proton NMR elemental analysis tAA-dUTP ($C_{12}H_{16}N_3O_{14}P_3Na_4.1H_2O$): theory C, 22.91; H, 2.88; N, 6.68; P, 14.77. Found, C, 23.10; H, 2.85; N, 6.49; P, 14.75. AA-UTP ($C_{12}H_{16}N_3O_{15}P_3Na_4.4H_2O$). Theory, C 20.61; H, 3.46; N, 6.01; P, 13.3. Found C, 20.67; H, 4.11; N, 5.39; P, 13.54] spectrally and chromatographically.

c) Biotination of AA-dUTP or AA-UTP

Biotinyl-N-hydroxysuccinimide ester (NHSB) was prepared from biotin (Sigma) as described previously (H. Heitzmann and F. M. Richards, Proc. Natl. Acad. Sci. USA. 71, 3537 [1974]). AA-dUTP-H_2O (63 mg, 0.1 mmole) or AA-UTP-$4H_2O$ (70 mg, 0.1 mmole) was dissolved in 20 ml of 0.1M sodium borate buffer at pH 8.5, and NHSB (34.1 mg, 0.1 mmole) dissolved in 2 ml of dimethyl formamide, was added. The reaction mixture was left at room temperature for four hours and then loaded directly onto a 30 ml column of DEAE-Sephadex TM A-25, preequilibrated with 0.1M TEAB at pH 7.5. The column was eluted with a 400 ml linear gradient (0.1-0.9M) of TEAB. Fractions containing biotinyl-dUTP or biotinyl-UTP, which eluted between 0.55 and 0.65M TEAB, were desalted by rotary evaporation in the presense of methanol and redissolved in water. Occasionally a slightly cloudy solution was obtained: this turbidity, due to a contaminant in some TEAB solutions, was removed by filtration through a 0.45 mm filter. For long term storage, the nucleotides were converted to the sodium salt by briefly stirring the solution in the presence of Dowex TM 50 (Na^+ form). After filtration the nucleotide was precipitated by the addition of three volumes of cold ethanol, washed with ethyl ether, dried in vacuo over sodium hydroxide pellets, and stored in a dessicator at −20° C. For immediate use, the nucleotide solution was made 20 mM in Tris-HCl at pH 7.5, and adjusted to a final nucleotide concentration of 5 mM. Stock solutions were stored frozen at −20° C.

Elemental analysis of the bio-dUTP and bio-UTP products yielded the following results, Bio-dUTP ($C_{22}H_{30}N_5O_{18}P_3S_1Na_6.1H_2O$). Theoretical; C, 29.80; H, 3.38; N, 7.89; P, 10.47; S, 3.61. Found, C, 30.14 H, 3.22; N, 7.63; P, 10.31; S, 3.70. Bio-UTP ($C_{22}H_{30}N_5O_{19}P_3S_1Na_4.3H_2O$): Theoretical; C, 29.15; H, 3.19; N, 7.45; P, 9.89; S, 3.41. Found; C, 28.76; H, 3.35; N, 7.68; P, 9.81; S, 3.32.

The spectral properties of bio-dUTP and bio-UTP at pH 7.5 [λ max, 289 nm (ε = 7,100); λ max, 240 nm (ε = 10,700); λ min, 262 nm (ε = 4,300)] reflect the presence of an exocyclic double-bond in conjugation with the pyrimidine ring. These nucleotides also give a strong positive reaction (an orange-red color) when treated with p-dimethylaminocinnamaldehyde in ethanolic sulfuric acid, a procedure used for biotin quantita-

tion (D. B. McCormick and J. A. Roth, Anal Biochem., 34,326, 1970). However, they no longer react with ninhydrin, a characteristic reaction of the AA-dUTP and AA-UTP starting materials.

EXAMPLES 3 and 4

Synthesis of biotinyl-CTP and biotinyl-dCTP

CTP and dCTP were a) mercurated, b) reacted with allylamine, and c) biotinized with NHS-biotin, essentially as described in Example 1. CTP (56.3 mg, 0.1 mmole) or dCTP (59.1 mg, 0.1 mmole) were dissolved in 20 ml of 0.1M sodium acetate buffer at pH 5.0, and mercuric acetate (0.159 gm, 0.5 mmoles) added. The solution was heated at 50° C. for 4.5 hours then cooled on ice. Lithium chloride (39.2 mg, 0.9 mmoles) was added and the solution extracted 6 times with ethyl acetate. The nucleotide products in the aqueous layer were precipitated by the addition of three volumes of cold ethanol and the precipitate collected by centrifugation. The precipitate was washed with absolute ethanol, ethyl ether, and then air dried. These products were used without further purification for the synthesis of AA-CTP and AA-dCTP, respectively. The mercurated nucleotides were dissolved in 0.1M sodium acetate buffer at pH 5.0 and adjusted to a concentration of 10 mM (92 OD/ml at 275 nm). 0.6 ml (1.2 mmole) of a 2.0M allylamine acetate stock (prepared as described in Example 1) was added to 10 ml of nucleotide solution (0.1 mmole) followed by the addition of K_2PdCl_4 (32.6 mg, 0.1 mmole), dissolved in 1.0 ml of H_2O. After standing at room temperature for 24 hours, the solution was filtered through a 0.45 mM membrane to remove metal precipates. The filtrate was diluted fivefold and loaded onto a 50 ml-column of DEAE-sephadex A-25, preequilibrated with 50 mM TEAB at pH 7.5. The nucleotide products were fractionated by application of a 500 ml linear gradient (0.05–0.6M) of TEAB at pH 7.5. The desired product was in the major UV absorbing portion which eluted between 0.28 and 0.38M salt. The pooled samples were desalted by rotary evaporation, dissolved in 0.5M triethylammonium acetate at pH 4.2, and final purification achieved by HPLC chromatography on columns of Partisil ODS-2, using 0.5M triethylammonium acetate as the eluent. Appropriate fractions were pooled, lyophilized, and the products dissolved in H_2O. The nucleotides were converted to the Na^+ salt by stirring briefly in the presence of Dowex TM 50 (Na^+ form). After filtration, to remove the Dowex resin, the nucleotides were precipitated by the addition of 3 volumes of cold ethanol. The precipitate was washed with ether and then air dried. Analytical results: AA-dCTP ($C_{12}H_{17}N_4O_{13}P_3Na_4.2H_2O$): Theory, C, 22.29; H, 2.63; N, 8.67; P, 14.40. Found C, 22.16; H, 2.89; N, 8.77; P, 14.18. AA-CTP ($C_{12}H_{17}N_4O_{14}Na_4.2H_2O$): Theory C, 21.75; H, 2.57; N, 8.46; P, 14.01. Found, C, 22.03; H, 2.47; N, 8.69; P, 13.81; Spectral properties in 0.1M Borate buffer at PH 8.0, λ max 301 nm (ε=6,400), λ min 271 nm (ε=3,950) λ max 250 nm (ε=9,700). Both AA-dCTP and AA-CTP give a positive ninhydrin test. AA-CTP (6.6 mg, 0.01 mmole) or AA-dCTP (6.4 mg, 0.01 mmole) was dissolved in 5 ml of 0.1M sodium borate buffer at pH 8.5, and NHS-biotin (3.4 mg, 0.01 mmole), dissolved in 0.2 ml of dimethylformamide, was added. After sitting at room temperature for 4 hours the sample was chromatographed on a 10 ml column of DEAE-Sephadex A-25, using a 150 ml linear gradient (0.1–0.9M) of TEAB at pH 7.5, as eluent. Fractions containing biotinyl-CTP or biotinyl-dCTP, which eluted between 0.50 and 0.60M TEAB, were pooled, desalted by rotary evaporation, and after being adjusted to a final concentration of 5 mM in 0.0M Tris-HCl buffer at pH 7.5, were frozen at −20° C. The products give a strong positive reaction for biotin with p-dimethylaminocinnamldehyde in ethanolic sulfuric acid but give a negative test for primary amines when sprayed with ninhydrin. Further structural characterization of these products is in progress.

EXAMPLES 5 and 6

Synthesis of Iminobiotinyl-UTP and Iminobiotinyl-dUTP

Iminobiotin hydrobromide was prepared from biotin as described previously (K. Hofmann, D. B. Melville and V. du Vigneaud, J. Biol. Chem., 141, 207–211, 1941; K. Hofmann and A. E. Axelrod, Ibid., 187., 29–33, 1950). The N-hydroxysuccinimide (NHS) ester of iminobiotin was prepared using the protocol previously described for the synthesis of NHS-Biotin (H. Heitzmann and F. M. Richards, Proc. Nat. Acad. Sci. USA, 71, 5537, 1974). AA-UTP (7.0 mg, 0.01 mmole) or AA-dUTP (6.3 mg, 0.01 mmole), prepared as detailed in example 1 (part b), was dissolved in 5 ml of 0.1M sodium borate buffer at pH 8.5, and NHS-iminobiotin (3.5 mg, 0.01 mmole), dissolved in 0.5 ml of dimethylformamide, was added. The reaction mixture was left at room temperature for 12 hours and then loaded directly onto a 10 ml column of DEAE-Sephadex A-25, preequilibrated with 0.05M TEAB at pH 7.5. The column was eluted with a 150 ml linear gradient (0.05–0.6M) of TEAB. Fractions containing iminobiotin-UTP or iminobiotin-dUTP, which eluted between 0.35 and 0.40M TEAB, were desalted by rotary evaporation in the presence of methanol and dissolved in H_2O. The products contained a small amount of allylamine-nucleotide adduct as an impurity, as judged by a weak positive result in the ninhydrin test. Final purification was achieved by affinity chromatography on avidinsepharose. Fractions of the impure product, made 0.1M in sodium borate buffer at pH 8.5, were applied to a 5 ml column of avidin-sepharose and washed with 25 ml of the same buffer. The column was then washed with 50 mM ammonium acetate buffer at pH 4.0, which eluted the desired iminobiotin-nucleotide product in a sharp peak. The nucleotide was precipitated by the addition of 3 volumes of cold ethanol, washed with ethylether, dried in vacuo over sodium hydroxide pellets and stored in a dessicator at −20° C. Products were characterized by elemental analysis, as well as by spectral and chromotographic properties.

EXAMPLES 7 and 8

Synthesis of NAGE-UTP and NAGE-dUTP

Allyl (3-ammo-2-hydroxy-)propyl ether, abbreviated MAGE, was prepared from allyl glycidyl ether (Age) (obtained from Aldrich Chemical Co.). 10 ml of Age (84 mmole) was added slowly (in a fume hood) to 50 ml of 9M ammonium hydroxide and the mixture allowed to stand at room temperature for six hours. Excess ammonia was removed by rotary evaporation under reduced pressure to yield a viscous yellow oil. Analysis of this product by proton NMR showed that it possessed the required structure. 5-mercuri-dUTP (0.1 mmole) or 5-mercuri-UTP (0.2 mmole) was dissolved in 2–4 ml of 0.2M sodium acetate buffer at pH 5.0, and a 16 fold

molar excess of NAGE adjusted to pH 5.0 with acetic acid prior to use, was added. The final reaction volumes (4.3 and 8.4 ml) had nucleotide concentrations of 43 and 42 mM, respectively. One equivalent of K_2PdCl_4 (0.1 or 0.2 mmoles) was added to initiate the reaction. After standing at room temperature for 18 hours, the reaction mixtures were filtered through 0.45 μmM membranes the samples diluted five-fold, and chromatographed on columns of DEAE-Sephadex A-25, using linear gradients (0.1–0.6M) of sodium acetate. Fractions containing the desired products, as judged by their UV spectra and characteristic HPLC elution profiles on Partisil ODS-2, were pooled, diluted, and further purified by re-chromatography on DEAE-Sephadex using shallow gradients (0.1-0.5M) of ammonium bicarbonate at pH 8.5. Under these conditions the majority of the NAGE-dUTP (or NAGE-UTP)could be cleanly separated from residual impurities. Proton NMR spectra were obtained at this stage of purification after the nucleotides were lyophilized and redissolved in D_2O. For elemental analysis, the products were converted to their sodium salt form. Typical analytical results: NAGE-dUTP ($C_{15}H_{22}N_3O_{16}P_3Na_4.2\ H_2O$). Theory, C, 24.99; H, 3.63; N, 5.83; P, 12.88. Found, C, 25.39; H, 3.71; N, 5.63; P, 12.88.

EXAMPLE 9

Uses of Labeled DNA Sequences

1. Karyotyping

(a) Select from a human gene library some 100 to 200 clones. Label them as described above, and for each clone locate its place or places of hybridization visually or with a low-light-level video system. For those clones which correspond to a unique sequence gene this determines the location of the cloned DNA on a particular human chromosome. Obtain several clones for each chromosome. Each of these labeled clones can be used to identify particular chromosomes. They can also be used in combination to identify each of the 46 chromosomes as being one of the 22 autosomal pairs or the X or the Y. By allowing one set of labeled clones to hybridize to the chromosomes and then adding a fluorescent stain to the label, the set of clones and their locations can be visualized and will fluoresce with a particular color. A second set of labeled clones could then be used and reacted with a second fluorescent dye. The same process can be repeated a number of times. Thus one can, if desired, have several sets of fluorescent labels attached to the cellular DNA at different but specific locations on each of the chromosomes. These labels could be used for visual or computerized automatic karyotyping.

(b) For automatic karyotyping, one could use one set of clones to identify the approximate location of each of the 46 chromosomes by finding sets of spots corresponding to the number of labeling sites on each chromosome. Thus, it is possible by computer analysis of the digitized images to determine if the chromosomes are suitably spread for further analysis. If they are suitably spread, then one can use computer analysis to identify each of the individual chromosomes by the location and distribution of the labelled spots on each one.

By using the fact that the fluorescent spots can be placed at specific locations on each chromosome, one can carry out either manual or automatic karyotyping very much more effectively than without such labels.

II. Diagnosis of Genetic Disorders

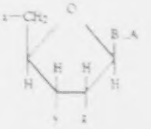
By selecting the clones which bind specifically to a particular chromosome, such as number 23, it is possible to count the number of copies of the particular chromosome in a cell even if the chromosomes are not condensed at metaphase. Thus when fetal cells are obtained for prenatal diagnosis of trisomy 21, the diagnosis can be done even if the chromosomes are not condensed at metaphase. If necessary, two sets of labels can be used—one which would be specific for chromosome 23 and one for some other chromosome. By measuring in each cell the ratio of the two labels, which might be of different colors, it is possible to identify the cells which show an abnormal number of chromosomes number 23. This procedure could be used either on slides with a low-light-level video system or in a flow cytometer system using laser excitation. It can be used to determine any abnormal chromosome number.

III. Microorganism Detection and Identification

The labeling of specific sequences of DNA as described above permits identification and counting of individual bacteria. In order to identify the individual bacteria to which a particular fragment of DNA hybridizes the sensitivity must be such that a single labelled structure can be detected. This can be done using a low-light-level video system and computer summation of images, or by using some other device for intensifying the light image. A flow system can also be used if the sensitivity can be made sufficiently grand. If one immobilized the bacteria on a slide their location could be found and the number of such fluorescent spots counted. This would provide a count of all of those bacteria which contain DNA which can hybridize with the specific clone utilized. If the clone is selected as being specific for a particular strain or bacteria, then one can count the number of organisms of that strain. In addition,any antibiotic resistance for which a particular gene has been identified could be characterized in a similar way using, as a probe, the DNA sequence which is contained in the antibiotic resistance gene. In addition,a probe could be used which is specific for a resistance plasmid containing one or more antibiotic resistance genes. In addition to individual bacteria, groups of bacterial cells of a particular strain can be detected and their number estimated if they are located in a small spot so that the total fluorescence specific to the hybridized DNA in the spot can be measured. In this way the number of organisms containing a specific DNA sequence can be measured in a mixture of bacteria.

We claim:

1. An oligo- or polynucleotide containing a nucleotide having the structure

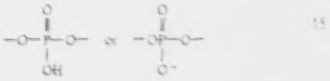


wherein B represents a 7-deazapurine or a pyrimidine moiety covalently bonded to the C1'-position of the sugar moiety, provided that whenever B is a 7-deazapurine, the sugar moiety is attached at the N9-position of the 7-deazapurine, and whenever B is a pyrimidine, the sugar moiety is attached at the N1-position of the pyrimidine,

wherein A comprises at least three carbon atoms and represents at least one component of a signaling moiety capable of producing a detectable signal.

wherein B and A are covalently attached directly or through a linkage group that does not substantially interfere with the characteristic ability of the oligo- or polynucleotide to hybridize with a nucleic acid and does not substantially interfere with formation of the signalling moiety or detection of the detectable signal, provided also that if B is 7-deazapurine, A or the linkage group is attached to the 7-position of the deazapurine, and if B is pyrimidine, A or the linkage group is attached to the 5-position of the pyrimidine;

wherein one of x and y represents



and the other of x and y is absent or represents —OH or —H; and

wherein z represents H— or HO—.

2. An oligo- or polynucleotide of claim 1 wherein B is selected from the group consisting of uracil, cytosine, deazaadenine, and deazaguanine.

3. An oligo- or polynucleotide of claim 1 wherein A is a ligand.

4. An oligo- or polynucleotide of claim 1 wherein A is a hapten.

5. An oligo- or polynucleotide of claim 4 wherein A is biotin.

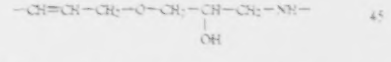
6. An oligo- or polynucleotide of claim 4 wherein A is iminobiotin.

7. An oligo- or polynucleotide of claim 1 wherein the linkage group includes an olefinic bond at the α-position relative to B.

8. An oligo- or polynucleotide of claim 1 wherein the linkage group includes the moiety —CH_2—NH—.

9. An oligo- or polynucleotide of claim 7 wherein the olefinic linkage group is the moiety —CH=CH—CH_2—NH—.

10. An oligo- or polynucleotide of claim 7 wherein the wherein the olefinic linkage group is



11. An oligo- or polynucleotide of claim 1 wherein A is an organic moiety containing at least five carbon atoms.

12. An oligo- or polynucleotide of claim 1 wherein z is HO—.

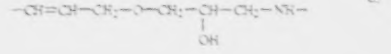
13. An oligo- or polynucleotide of claim 1 wherein z is H—.

14. An oligo- or polynucleotide of claim 11 wherein A is biotin.

15. An oligo- or polynucleotide of claim 11 wherein A is iminobiotin.

16. An oligo- or polynucleotide of claims 14 or 15 wherein the linkage group is —CH=CH—CH_2—NH—.

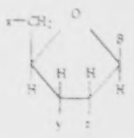
17. An oligo- or polynucleotide of claim 14 or 15 wherein the linkage group is



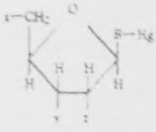
18. An oligo- or polynucleotide of claim 14 or 15 wherein B is uracil.

19. A method of making the oligo- or polynucleotide of claim 1 comprising the steps of:

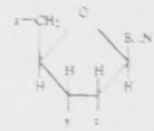
(a) reacting an oligo- or polynucleotide containing a nucleotide having the structure



with a mercuric salt in a suitable solvent under suitable conditions so as to form a mercurated derivative compound having the structure



(b) reacting said mercurated derivative compound with a chemical moiety reactive with the —Hg+ portion of said mercurated compound and represented by the formula —N, said reaction being carried out in an aqueous solvent and in the presence of a palladium-containing compound under suitable conditions so as to form a compound having the structure



wherein N is a reactive terminal functional group or is A; and

(c) when N is A, recovering said oligo- or polynucleotide of claim 1; or when N is a reactive terminal group, reacting said compound with a compound having the structure M—A wherein M represents a functional group reactive with N in an aqueous solvent under suitable conditions so as to form said oligo- or polynucleotide of claim 1 and recovering said oligo- or polynucleotide.

20. A method in accordance with claim 19 wherein said suitable conditions comprise a pH in the range from about 4 to 8, a temperature in the range from about 20° C. to 30° C., and a reaction time in the range from about 3 to 24 hours.

21. A method in accordance with claim 19 wherein said mercuric salt is mercuric acetate.

22. A method in accordance with claim 19 wherein said chemical moiety represented by the formula —N is

—CH=CH—CH_2—NH$_2$

23. A method in accordance with claim 19 wherein said chemical moiety represented by the formula ... N is

24. A method in accordance with claim 19 wherein said aqueous solvents include a buffer.

25. A method in accordance with claim 24 wherein said buffer is selected from the group consisting of sodium acetate, potassium acetate, sodium citrate, potassium citrate, potassium citrate-phosphate, tris-acetate, and borate-sodium hydroxide.

26. A method in accordance with claim 25 wherein the concentration of said buffer is less than about 2.0 molar.

27. A method in accordance with claim 19 wherein in step (b) the aqueous solvent additionally includes an organic solvent.

28. A method in accordance with claim 27 wherein said organic solvent is water-miscible.

29. A method in accordance with claim 27 wherein said organic solvent is selected from the group consisting of alcohols, ketones, and amides.

30. A method in accordance with claim 29 wherein said organic solvent is selected from the group consisting of methanol, ethanol, propanol, glycerin, dioxane, acetone, pyridine, and dimethylformamide.

31. A method in accordance with claim 29 wherein A comprises biotin or iminobiotin, and M is selected from the group consisting of N-hydroxy-succinimide ester, imidate, anhydride, isothiocyanate, and epoxide.

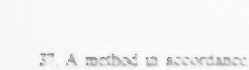
32. A method in accordance with claim 19 wherein M is an N-hydroxysuccinimide ester.

33. A method in accordance with claim 19 wherein M is selected from the group consisting of imidate, anhydride, isothiocyanate, and epoxide.

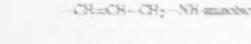
34. A method in accordance with claim 19 wherein N is selected from the group consisting of thiol, carboxylic acid, epoxide, and amine.

35. A method in accordance with claim 19 wherein said chemical moiety represented by the formula ... N is

36. A method in accordance with claim 19 wherein said chemical moiety represented by the formula ... N is



37. A method in accordance with claim 19 wherein said chemical moiety represented by the formula ... N is



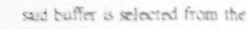
38. A method in accordance with claim 19 wherein said chemical moiety represented by the formula ... N is

39. A double-stranded polynucleotide duplex which includes the oligo- or polynucleotide of claim 1.

40. The duplex of claim 39 wherein said polynucleotide strands are ribonucleic acid molecules.

41. The duplex of claim 39 wherein said polynucleotide strands are deoxyribonucleic acid molecules.

42. An oligo- or polynucleotide sequence comprising at least one of a moiety having the structure:



wherein B represents a 7-deazapurine or a pyrimidine moiety suitable for incorporation into a polynucleotide and;

wherein A comprises at least three carbon atoms and represents at least one component of a signalling moiety capable of producing a detectable signal;

wherein B and A are covalently attached directly or through a linkage group that does not substantially interfere with the characteristic ability of the sequence to hybridize with a nucleic acid and does not substantially interfere with formation of the signalling moiety or detection of the detectable signal, provided also that if B is 7-deazapurine, A or the linkage group is attached to the 7-position of the deazapurine, and if B is pyrimidine, A or the linkage group is attached to the 5-position of the pyrimidine.

43. An oligo- or polynucleotide sequence of claim 42 wherein A is a ligand.

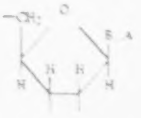
44. An oligo- or polynucleotide sequence of claim 42 wherein A is a polypeptide.

45. An oligo- or polynucleotide sequence wherein A is fluorescent, electron dense, or is an enzyme capable of reacting with a substrate to form a detectable reaction product.

46. An oligo- or polynucleotide sequence of claim 42 wherein A is a moiety consisting of at least five carbon atoms.

47. An oligo- or polynucleotide sequence of claim 42 wherein B is a pyrimidine.

48. An oligo- or polynucleotide containing therein a sugar moiety having the structure

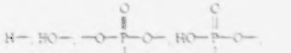


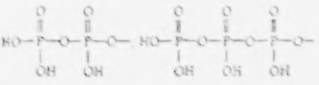
wherein B represents a 7-deazapurine or a pyrimidine moiety covalently bonded to the C^1-position of the sugar moiety, provided that whenever B is a 7-deazapurine, the sugar moiety is attached at the N^9-position of the 7-deazapurine, and whenever B is a pyrimidin, the sugar moiety is attached at the N^1-position of the pyrimidine;

wherein A comprises at least three carbon atoms and represents at least one component of a signaling moiety capable of producing a detectable signal; and

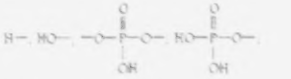
wherein B and A are covalently attached directly or through a linkage group that does not substantially interfere with the characteristic ability of the oligo- or polynucleotide to hybridize with a nucleic acid and does not substantially interfere with formation of the signalling moiety or detection of the detectable signal, provided also that if B is 7-deazapurine, A or the linkage group is attached to the 7-position of the deazapurine, and if B is pyrimidine, A or the linkage group is attached to the 5-position of the pyrimidine.

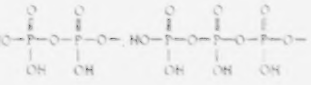
49. An oligo- or polynucleotide of claim 48, wherein the sugar moiety has attached to its C^3-position a member selected from the group consisting of





50. An oligo- or polynucleotide of claim 48, wherein the sugar moiety has attached to its C^5-position a member selected from the group consisting of





51. An oligo- or polynucleotide of claim 48, wherein the sugar moiety has attached to its C^2-position a H— or HO— group.

52. A method in accordance with claim 19, wherein the palladium-containing compound is K_2PdCl_4.

53. An oligo- or polynucleotide of claim 48 wherein A is a ligand.

54. An oligo- or polynucleotide of claim 3 or 53 wherein the ligand is selected from the group consisting of a hapten, an antigen, a cofactor, biotin and iminobiotin.

55. An oligo- or polynucleotide of claim 3 or 53 wherein the ligand is selected from the group consisting of dinitrophenol, lipoic acid and an olefinic compound.

56. An oligo- or polynucleotide of claim 3 or 53 wherein the ligand is capable of forming a complex by binding with a detectable polypeptide.

57. An oligo- or polynucleotide of claim 56 wherein the detectable polypeptide is selected from the group consisting of an antibody, an enzyme, streptavidin and avidin.

58. An oligo- or polynucleotide of claim 56 wherein an indicator molecule is associated with or bound to the detectable polypeptide.

59. An oligo- or polynucleotide of claim 58 wherein the indicator molecule is fluorescent, electron dense, or an enzyme capable of depositing insoluble reaction products.

60. An oligo- or polynucleotide of claim 59 wherein the enzyme is selected from the group consisting of alkaline phosphatase, peroxidase and β-galactosidase.

61. An oligo- or polynucleotide of claim 59 wherein the fluorescent indicator molecule is selected from the group consisting of fluorescein and rhodamine.

62. An oligo- or polynucleotide of claim 59 wherein the electron dense indicator molecule is selected from the group consisting of ferritin, hemocyanin and colloidal gold.

63. An oligo- or polynucleotide of claim 58 wherein the indicator molecule is covalently linked to the detectable polypeptide.

64. An oligo- or polynucleotide of claim 58 wherein the detectable polypeptide is indirectly detectable by specifically complexing the detectable polypeptide with a second polypeptide covalently linked to an indicator molecule.

65. An oligo- or polynucleotide of claim 64 wherein said detectable polypeptide is selected form the group consisting of avidin and streptavidin and the second polypeptide is selected from the group consisting of biotin and iminobiotin.

66. An oligo- or polynucleotide of claim 56 wherein at least one compound is labeled with a first indicator molecule and at least one other compound is labeled with a second indicator molecule.

67. An oligo- or polynucleotide of claim 1 or 48 wherein A comprises an indicator molecule.

68. An oligo- or polynucleotide of claim 67 wherein said indicator molecule is fluorescent, electron dense, or is an enzyme capable of depositing insoluble reaction products.

69. An oligo- or polynucleotide of claim 68 wherein the enzyme is selected from the group consisting of alkaline phosphatase, peroxidase and β-galactosidase.

70. An oligo- or polynucleotide of claim 68 wherein the fluorescent indicator molecule is selected from the group consisting of fluorescein and rhodamine.

71. An oligo- or polynucleotide of claim 68 wherein the electron dense compound is selected from the group consisting of ferritin, hemocyanin and colloidal gold.

* * * * *

APPENDIX B

SUPPLEMENTAL AMENDMENT OF MARCH 21, 1986, U.S. PATENT APPLICATION SERIAL NO. 496,915

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner	:	Ethel G. Love
Group	:	123
Applicants	:	David C. Ward et al.
Serial No.	:	496,915
Filed	:	May 23, 1983
For	:	MODIFIED NUCLEOTIDES AND METHODS OF PREPARING AND USING SAME

New York, New York
March 21, 1986

Hon. Commissioner of Patents
and Trademarks
Washington, D.C. 20231

SUPPLEMENTAL AMENDMENT

Sir:

Kindly amend the above-identified application as follows:

IN THE CLAIMS

Please amend claims 145, 149, 150, 155, 163, 164, 166, 172, 173, 183, 191, 192 and 199.

145. (four times amended) A nucleotide or oligo- or polynucleotide sequence comprising at least one of a moiety having the structure:

-BA

wherein B represents a 7-deazapurine or a pyrimidine moiety;

wherein A [comprises] represents a moiety selected from the group consisting of biotin and iminobiotin;

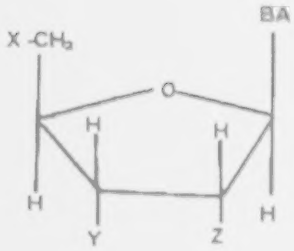
provided that if B is a 7-deazapurine, A is attached to the 7-position of the deazapurine, and if B is a pyrimidine, A is attached to the 5-position of the pyrimidine, A being attached to B directly or through a linkage group said linkage group not interfering substantially with the characteristic ability of A to form a stable complex with avidin.

149. (four times amended) [A] The nucleotide or oligo- or polynucleotide sequence according to [claim] Claim 145, wherein [said A also comprises a second moiety selected from the group consisting of a moiety including] said linkage group is characterized by at least one of an olefinic bond at the α-position relative to B and a [moiety including] $-CH_2NH-$ [, the second moiety linking B to the biotin or iminobiotin].

150. (twice amended) The nucleotide or oligo- or polynucleotide sequence in accordance with Claim 149, wherein the linkage group is characterized by [second moiety includes] the moiety $-CH=CH-CH_2-NH-$ or

$$-CH=CH-CH_2-O-CH_2-\underset{\displaystyle OH}{\underset{|}{CH}}-CH_2-NH-$$

155. (four times amended) A compound having the structure:

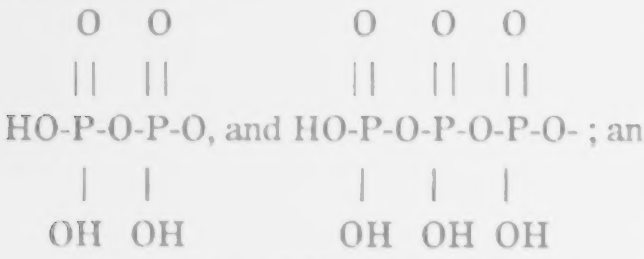


wherein B represents a 7-deazapurine or a pyrimidine moiety covalently bonded to the $C^{1'}$- position of the sugar moiety, provided that when B is a 7-deazapurine, it is attached to the N^9-position of the deazapurine, and when B is a pyrimidine, it is attached at the N^1-position of the pyrimidine;

wherein A [comprises] represents a moiety selected from the group consisting of biotin and iminobiotin;

provided that if B is a 7-deazapurine, A is attached to the 7-position of the deazapurine, and if B is a pyrimidine, A is attached to the 5-position of the pyrimidine, A being attached to B directly or through a linkage group, said linkage group not interfering substantially with the characteristic ability of A to form a stable complex with avidin;

wherein X represents a moiety selected from the group consisting of:



; and

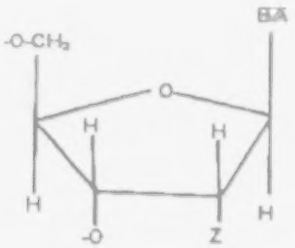
wherein Y is -OH and Z is -OH or -H.

163. (three times amended) The compound [is] in accordance with Claim 155, wherein [said A also comprises a second moiety selected from the group consisting of a moiety including] said linkage group is characterized by at least one of an olefinic bond at the α-position relative to B and a [moiety including] $-CH_2NH-$ [, the second moiety linking B to the biotin or iminobiotin].

164. (twice amended) The compound in accordance with Claim 163, wherein the linkage group is characterized by [second moiety includes] a moiety selected from the group consisting of $-CH=CH-CH_2-NH-$ and

$$-CH=CH-CH_2-O-CH_2-\underset{\displaystyle OH}{\underset{|}{CH}}-CH_2-NH-$$

166. (four times amended) A poly- or oligonucleotide sequence which comprises at least one of a first moiety having the structure:

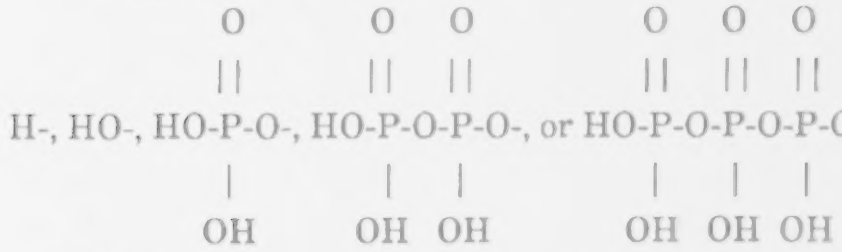


wherein B represents a purine, a 7-deazapurine or a pyrimidine moiety covalently bonded to the $C^{1'}$-position of the sugar moiety, provided that when B is a purine or a 7-deazapurine, it is attached to the N^9-position of the purine or deazapurine, and when B is a pyrimidine, it is attached at the N^1-position of the pyrimidine;

wherein A [comprises] represents a moiety selected from the group consisting of biotin and iminobiotin;

provided that if B is a purine, A is attached to the 8-position of the purine, if B is a 7-deazapurine, A is attached to the 7-position of the deazapurine, and if B is a pyrimidine, A is attached to the 5-position of the pyrimidine, A being attached to B directly or through a linkage group, said linkage group not interfering substantially with the characteristic ability of A to form a stable complex with avidin; and

wherein Z represents:

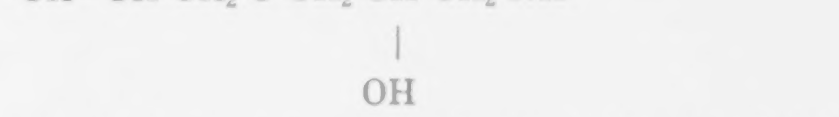


H-, HO-, HO-P-O-, HO-P-O-P-O-, or HO-P-O-P-O-P-O-

OH OH OH OH OH OH OH

172. (three times amended) The sequence in accordance with Claim 166, wherein [said A also comprises a second moiety selected from the group consisting of a moiety including] said linkage group is characterized by at least one of an olefinic bond at the α-position relative to B and a [moiety including] $-CH_2-NH-$[, the second moiety linking B to the biotin or iminobiotin].

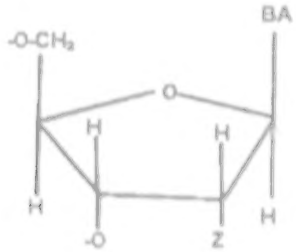
173.(twice amended) The sequence in accordance with Claim 172, wherein the linkage group is characterized by [second moiety includes the] a moiety selected from the group consisting of $-CH=CH-CH_2-NH-$ and



OH

183. (four times amended) A double-stranded RNA or DNA duplex or DNA-RNA hybrid which comprises:

(i) in one strand a poly- or oligonucleotide sequence which comprises at least one of a moiety having the structure:

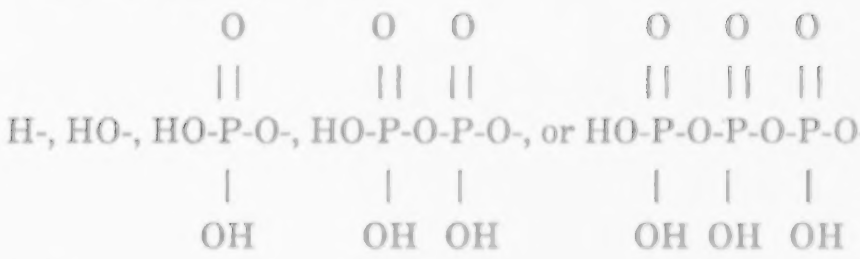


wherein B represents a purine, a 7-deazapurine or a pyrimidine moiety covalently bonded to the $C^{1'}$-position of the sugar moiety, provided that when B is a purine or a 7-deazapurine, it is attached at the N^9-position of the purine or deazapurine, and when B is a pyrimidine, it is attached at the N^1-positon of the pyrimidine;

wherein A represents [comprises] a moiety selected from the group consisting of biotin and iminobiotin;

provided that if B is a purine, A is attached to the 8-position of the purine, if B is a 7-deazapurine, A is attached to the 7-position of the deazapurine, and if B is a pyrimidine, A is attached to the 5-position of the pyrimidine, A being attached to B directly or through a linkage group, said linkage group not interfering substantially with the characteristic ability of A to form a stable complex with avidin; and

wherein Z represents



and

(ii) in the second strand, a poly- or oligonucleotide sequence containing neither a biotin or iminobiotin.

191. (three times amended) The duplex or hybrid in accordance with Claim 183, wherein [said A also comprises a second moiety selected from the group consisting of a moiety including] said linkage group is characterized by at least one of an olefinic bond at the α-position relative to B and a [moiety including] $-CH_2-NH-$.

192. (twice amended) The duplex or hybrid in accordance with Claim 191, wherein the linkage group is characterized by [second moiety includes] a moiety selected from the group consisting of $-CH=CH-CH_2-NH-$ and

199. (twice amended) A nucleotide or oligo- or polynucleotide sequence comprising at least one of a moiety having the structure:

-BA

wherein B represents purine;

wherein A [comprises] represents a [first] moiety selected from the group consisting of biotin and iminobiotin, attached to the 8-position of the purine, A being

attached to B through a linkage group, said linkage group not interfering substantially with the characteristic ability of A to form a stable complex with avidin [comprising] an olefinic bond at the α-position relative to B[, the second moiety linking B to the biotin or iminobiotin].

REMARKS

Applicants express their appreciation for the courtesy extended to their attorneys, James F. Haley, Jr., and Samuel B. Abrams, during a February 5, 1986 interview with Examiner Love. Applicants make that interview of record here.

At the interview the Examiner's rejections of Claims 145, 148-150, 155, 162-164, 166, 171-174, 177, 183, 184, 189-192 and 199 under 35 U.S.C. §112, paragraphs one and two, and applicants' February 3, 1986 Amendment in response were discussed.

With respect to the Examiner's contention (1) that the term "a compound" in Claims 145, 149 and 199 was "so broad that the scope of the claimed compound is indeterminable", applicants' attorneys pointed out that Claims 145, 149 and 199 as amended on February 3 avoided the objected to language. In view of those amendments, applicants' attorneys understood that the Examiner no longer maintained her rejection of Claims 145, 149, and 199, except to the extent referred below.

With respect to the Examiner's contention (2) that the definition of a linkage group "..." was absent from the claims, making their scope "indeterminable," applicants' attorneys pointed to their February 3 amendment of Claims 145, 149, 150, 155, 163, 164, 166, 172, 173, 183, 191, 192 and 199 omitting the objected to "..." terminology. Applicant' attorneys also stated that the amended claims

with their "comprising" and "including" terminology defined the invention by specific label -- biotin or iminobiotin -- and by specific position of attachment of that label to the base that characterizes the nucleotides and the oligo- and polynucleotides of the invention. Furthermore, they urged that the linkage group attaching the label to the base was optional and irrelevant to the invention and that substantially any linkage group could be used without departing from the scope of the disclosed invention. Applicants' attorneys also pointed out that the amended claims were no broader than the specification, that there was no art of record that anticipated those claims or made their subject matter obvious, and that the subject matter of those claims was operative and specifically exemplified in the specification. Examiner Love, although not contesting any of those facts, still considered the claims "open-ended and indefinite" because of their "comprising" and "including" terminology.

To speed the allowance of this patent application, applicants have again amended their claims to clarify the attachment of A to B. As amended, the claims avoid the "comprising" and "including" terminology objected to by Examiner Love and define the A to B attachment clearly and distinctly. The amended claims encompass nucleotides or oligo- or polynucleotide sequences in which the biotin or iminobiotin moiety (A) is attached to the base (B) either directly or through a linkage group which does not interfere substantially with the characteristic ability of A to form a stable complex with avidin. This language defines the optional linkage group in terms of a specific and unambiguous test – interference with A's characteristic ability to bind to avidin. In selecting this test to define the linkage group, applicants do not suggest that A can only bind to avidin. As described in the specifi-

cation, A can bind to a variety of polypeptides. However, for the purposes of a definition of useful linkage groups, only the binding of A to avidin need be considered.

Support for this amendment can be found in the specification, for example, on page 27, line 26-page 28, line 9 which describes the detection of the claimed compounds "by contacting the compounds with polypeptides [e.g., avidin] which are capable of forming complexes therewith under suitable conditions so as to form the complexes...." Further support is found at page 13, lines 10-31 and at page 14, lines 1-13 of the specification, where preferred linkage groups are specifically described.

The amended claims are no broader than the specification. They are also not anticipated or made obvious by any of the documents of record. And, the subject matter of the amended claims is operative and exemplified in the specification. Accordingly, applicants request that Examiner Love allow the amended claims.

With respect to the Examiner's contention (3) that claims encompassing deazapurine derivatives were "insufficiently disclosed in the specification", applicants' attorneys pointed to pages 6 and 17 of the specification and urged that the synthesis of the deazapurines was known to be analogous to that of pyrimidines. Accordingly, the specification adequately supports the claimed "deazapurines". It is applicants' attorneys' understanding that the Examiner has withdrawn her rejection of those compounds.

Finally, with respect to the Examiner's contention (4) that Claim 183 was indefinite and too broad, applicants' attorneys pointed out that the amended claims had omitted the objected to "to be discriminated" or "during

detection" language. As a result, the Examiner withdrew her rejection.

In summary, as a result of their February 3 amendments (contentions 3 and 4) and the above amendments (contentions 1 and 2), applicants have avoided and resolved all of Examiner Love's rejections under 35 U.S.C. §112. The amended claims are now in condition for allowance.

With respect to the Examiner Love's rejection of Claims 145, 148-150, 155, 162-164, 166, 171-174, 177, 183, 184, 189-192 and 192 as unpatentable under 35 U.S.C. §103, applicants' attorneys discussed at the February 5 interview the various cited documents* and argued that none taught or suggested the combination of applicants' labels and their positions of labelling nucleotides. As a result, the Examiner agreed that the "references of record are not seen to show the claimed invention," except as that invention is claimed in claims 145 and 148-150.

Applicants request Examiner Love to reconsider her rejection of Claims 145 and 148-150. The subject matter of amended claims 145, 148-150 are not taught or suggested by the art. For example, A. Sodja and N. Davidson, "Gene Mapping And Gene Enrichment By the Avidin-Biotin Interaction: Use of Cytochrome-C As A Polyamine Bridge," Nucl. Acids Res., 5, pp. 383-99 (1978)

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* United Kingdom Patent 2,019,408 ("Kourilsky"); Manning et al., "A New Method of In Situ Hybridization," Chromosoma, 53, pp. 107-17 (1975) ("Manning"); Ruth et al., "C-5 Substituted Pyrimidine Nucleosides," J. Org. Chem., 43 (14), pp. 2870-76 (1978) ("Ruth"); Bayer et al., "The Use of The Avidin Biotin Complex As A Tool In Molecular Biology," Methods of Biochemical Analysis, 26, pp. 1-45 (1980) ("Bayer"); and Stryer, Biochemistry (2d Ed.), pp. 348, 512, 589 and 596 (1981) ("Stryer").

("Sodja"), ** pointed to by Examiner Love, refers only to methods of coupling biotin through a cytochrome C bridge to the oxidized 2', 3' terminus of an RNA sugar ring. None of Sodja's compounds suggests the nucleotides or oligo- or polynucleotide sequences of applicants' amended claims 145 and 148-150 because none of Sodja's compounds has a biotin substituent in the base position required in those claims. All of Sodja's compounds have a biotin substituent on the sugar moiety, not on the 5 or 7 position of the base moiety.

For example, applicants' Claim 145 (as amended), and claims dependent thereon, describe nucleotide sequences comprising at least one labelled pyrimidine or 7-deazapurine, wherein a biotin or iminobiotin moiety is attached, either directly or through a linkage group, to the 7-positon of the deazapurine or to the 5-position of the pyrimidine. Applicants' labelled nucleotides are not suggested by Sodja where biotin is attached to the 2', 3' oxidized positions of a sugar ring. Accordingly, amended Claims 145 and 148-150 are patentable over all the documents of record.

Applicants request early allowance of the amended claims.

Respectfully submitted

/s/

James F. Harley, Jr. (Reg. No. 27,794)
Irene J. Frangos (Reg. No. 31,252)
Attorneys for Applicants

** Applicants made this document of record in their Statement Under 37 C.F.R. §§ 1.56 and 1.97, filed June 24, 1985.

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New York, New York 10022-6250
Tel.: (212) 715-0600

APPENDIX C

OFFICE ACTION OF JULY 2, 1986, U.S. PATENT APPLICATION SERIAL NO. 496,915

EXAMINER'S ACTION

[Seal Omitted]

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.
06/496,915	05/23/83	Ward D	18571 A

JAMES F. HALEY, JR.
C/O FISH AND NEAVE
875 THIRD AVENUE 29TH FLOOR
NEW YORK, NY 10022

EXAMINER	
LOVE, E	
ART UNIT	PAPER NUMBER
123	[19]*

DATE MAILED: 07/02/86

This is a communication from the examiner in charge of your application.

COMMISSIONER OF PATENTS AND TRADEMARKS

☒ This application has been examined

☒ Responsive to communication filed on [2/4/86, 3/24/86]

☐ This action is made final.

A shortened statutory period for response to this action is set to expire [3] month(s), _____ [~~days~~] from the date of this letter. Failure to respond within the period for response will cause the application to become abandoned. 35 U.S.C. 133

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* All text in brackets was handwritten in the original.

Part I THE FOLLOWING ATTACHMENT(S) ARE PART OF THIS ACTION:

1. ☐ Notice of References Cited by Examiner, PTO-892
2. ☐ Notice re Patent Drawing, PTO-948
3. ☐ Notice of Art Cited by Applicant, PTO-1449
4. ☐ Notice of informal Patent Application, Form PTO-152
5. ☐ Information on How to Effect Drawing Changes, PTO-1474
6. ☐ _____

Part II SUMMARY OF ACTION

1. ☒ Claims [145, 148-150, 155, 162-164, 166, 171-174, 177, 183, 184, 189-192 and 199] are pending in the application.

   Of the above claims _____ are withdrawn from consideration.
2. ☐ Claims _____ have been cancelled.
3. ☐ Claims _____ are allowed.
4. ☒ Claims [145, 148-150, 155, 162-164, 166, 171-174, 177, 183, 184, 189-192 and 199] are rejected.

* * * * *

Serial Number 496,915

Art Unit 123

Claims 145, 148-150, 155, 162-164, 166, 171-174, 177, 183, 184, 189-192 and 199 are in the case.

Claims 145, 148-150, 155, 162-164, 166, 171-174, 177, 183, 184, 189-192 and 199 are again rejected under 35 U.S.C. 112, paragraphs one and two as being too broad with respect to the terminology or recitations "linkage group is characterized by a moiety", "a linkage group not interfering substantially with the characteristic ability of A to form a stable complex with avidin" and "linkage group is characterized by at least one of". [T]he recitations are still indefinite and indeterminable in scope. A markush recitation of linkage groups would be acceptable. See 37 CFR 1.75(d(1).

Claims 145, 148-150, 155, 162-164, 166, 171-174, 177, 183, 184, 189, 192 and 199 are rejected under 35 U.S.C. 102(a) as being anticipated by [and] 35 U.S.C. 103 as being obvious over the Langer et al (II) abstract 1153 and full publication of February 8 to March 20, 1981. The rejection is proper in that the publications are prior to applicants' effective date of April 17, 1981. The references disclose the instant claimed invention or su[b]ject maatter so closely related thereto that the claimed invention would be unpatentable thereover. [illegible handwritten note at the side of this paragraph]

E.G. Loves :dm
(703) 557-3412
June 27, 1986

/s/

Ethel G. Love
Primary Examiner
Art Unit 123

APPENDIX D

AMENDMENT OF DECEMBER 31, 1986, U.S. PATENT APPLICATION SERIAL NO. 496,915

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner	:	Ethel G. Love
Group	:	123
Applicants	:	David C. Ward et al.
Serial No.	:	496,915
Filed	:	May 23, 1983
For	:	MODIFIED NUCLEOTIDES AND METHODS OF PREPARING AND USING SAME

New York, New York
December 31, 1986

Hon. Commissioner of Patents
and Trademarks
Washington, D.C. 20231

AMENDMENT

Sir:

This is in response to the Office Action dated July 2, 1986 in the above-identified application. Applicants have petitioned concurrently herewith to extend the time for response to that Office Action up to and including January 2, 1987 and have paid the required fee [37 C.F.R. §§ 1.17 and 1.136(a)].

Applicants request amendment of this application as follows:

IN THE CLAIMS

Please amend claims 145, 155, 166, 183 and 199.

* * * * *

Applicants traverse the rejection of Claims 145, 148-50, 155, 162-164, 166, 171-174, 177, 183, 184, 189-192 and 199 under 35 U.S.C. § 112, paragraphs 1 and 2.

The definition of "linkage group" in the amended claims is in no way "indefinite or indeterminable in scope", but rather is finite with a definite boundary. It is fully commensurate with the teachings of this application. It is fully consistent with the use of the term "linking group" in the art. It is also fully supported by numerous actual examples in the application, by examples reported subsequently in the art and by examples actually performed in the laboratories of Enzo Biochem, Inc., the licensee of this application. For all of these reasons, no specific Markush definition of the linkage group is necessary in the amended claims. The amended claims are patentable under 35 U.S.C. § 112.

A compound is defined by its structure and function [In re Papesch, 315 F.2d 381, 137 USPQ 43 (CCPA 1963)]. In the biological sciences, it is well recognized that function is a critical element to describe fully a compound. Accordingly, applicants have provided definitions of both structure and function in their amended claims. The amended claims require a specific structure of a nucleotide, oligonucleotide or polynucleotide and a specific function to define the "linkage group" within the claimed structure. That function is no substantial interference with the characteristic ability of A (biotin or iminobiotin) to form a detectable complex with one of avidin, steptavidin or antibodies to biotin or iminobiotin. Thus, the

claims are fully commensurate with the requirements of the patent laws.

As made plain in this application, the claimed compounds of this invention are characterized by two important biological functions: (1) the oligo- and polynucleotide probes "labelled" with biotin or iminobiotin in accordance with this invention must be able to bind to complementary DNA sequences in an analyte; and (2) after such binding, the biotin or iminobiotin "label" must be detectable by complexing with one of avidin, streptavidin or antibodies to biotin or iminobiotin. The first of these biological functions is accomplished in the claimed invention by applicants' choice of specific labelling positions on a given base for attachment of the biotin or iminobiotin label.

The second of these essential biological functions is a characteristic of applicants' selection of two specific labels – biotin or iminobiotin. These labels have the property of forming detectable complexes with avidin, streptavidin or antibodies to the labels. It is this property that makes applicants' claimed compounds detectable as probes. See, e.g., page 22, line 35 - page 23, line 7:

> "It is clear from the data in these tables that extremely small amounts of biotin can be detected by the method. These results also show that the biotin molecule can be recognized by avidin, streptavidin or specific antibodies while the DNA is still in its native, double-stranded form, a condition that is absolutely essential if the antibody-binding or avidin-affinity approaches are to be useful in probe detection employing hybridization techniques". See also page 9, line 32-page 10, line 2 and page 27, line 26-page 29, line 10.

Thus, the only relevance of the "linkage group" to the claimed compounds and their use as probes is that the "linkage group" not interfere substantially with this property of the biotin and iminobiotin labels. Accordingly, there is no need to limit applicants' claims to one particular Markush group of linkers.

The teaching and disclosure of the application about useful linkers is certainly not limited to a Markush group. Rather, it is fully commensurate with the true scope of applicants' invention -- the biotin or iminobiotin label must be able to form a detectable complex with one of avidin, streptavidin or antibodies. For example, at page 9, line 32- page 10, line 2, the application describes the linker in similar terms to the amended claims:

> "[I]t is preferable that the probe moiety [biotin or iminobiotin] be attached to the purine or pyrimidine [the base] through a chemical linkage or 'linker arm' so that it can readily interact with antibodies, other detector proteins or chemicals reagents."

Apposite also are the many disclosures in the application with respect to the wide range of atoms and functionalities acceptable in a linkage group. E.g.:

> "The linkage group joining moiety A to base B may include any of the well-known bonds including carbon-carbon single bonds, carbon-carbon double bonds, carbon-nitrogen single bonds or carbon-oxygen single bonds" [page 13, lines 10-13].
>
> "[I]t is generally preferred that the chemical linkage include an olefinic bond at the α-position relative to B" [page 13, lines 14-15].
>
> "[Among linkage groups that can be used are] olefin linker arms with other modifiable functionalities

such as thiol, carboxylic acid, and epoxide functionalities" [page 14, lines 2-4].

"It is even more preferred that the chemical linkage group be derived from a primary amine, and have the structure -CH_2-NH-...." [page 13, lines 25-27].

Finally, the examples of specific linkage groups recited in the application confirm that the claimed invention is not limited to particular linkage groups. Those illustrative linkage groups include carbon, hydrogen, nitrogen and oxygen atoms. They also include carbon-carbon single and double bonds, carbon-nitrogen single bonds, carbon-hydrogen single bonds and carbon-oxygen single bonds. E.g., -CH=CH-CH_2-NH- and

$$CH=CH\text{-}CH_2\text{-}O\text{-}CH_2\text{-}\underset{\displaystyle OH}{\underset{|}{CH}}\text{-}CH_2\text{-}NH\text{-}$$

(page 18, line 31).

Thus, the application itself never suggests that the described invention is limited to a specific Markush group of linkers. There is, therefore, no reason for the claims to be narrower than that invention.

In addition to being completely supported by this application, the definition of "linkage group" in the amended claims is also fully consistent with a wide variety of illustrative linkage groups actually demonstrated or reported to be useful in preparing compounds within applicants' claims.

As suggested by Examiner Love during the July 30, 1986 interview, applicants now make of record eight of these linkage groups by listing them in Exhibit A to the Declaration Of Dean L. Engelhardt Under 37 C.F.R. § 1.132, filed concurrently herewith ("Engelhardt Declaration"). These linkage groups conform structurally and

functionally to the description in the specification and the definition of the amended claims. They contain carbon, nitrogen, hydrogen, oxygen and sulfur atoms. They contain carbon-carbon single and double bonds, carbon-nitrogen single bonds, carbon-oxygen single and double bonds, carbon-hydrogen single bonds, carbon-sulfur single bonds and sulfur-sulfur single bonds. They also contain heterocylic and aromatic rings. And, they include straight chains of from 4 to 16 atoms in length. Yet, none of these diverse linkers functionally interfere with the ability of biotin in an olgio- or polynucleotide probe of this invention to form a detectable complex with one of avidin, streptavidin or antibodies to biotin. The demonstrated consistent utility of this wide structural variety of linkage groups in the compounds of this invention supports a single conclusion -- there is no reason to limit the "linkage group" of applicants' claimed compounds to a single Markush group.

* * * * *

APPENDIX E

OFFICE ACTION OF APRIL 16, 1987, U.S. PATENT APPLICATION SERIAL NO. 496,915

EXAMINER'S ACTION

[Seal Omitted]

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.
06/496,915	05/23/83	Ward D	18571-A

JAMES F. HALEY, JR.
C/O FISH AND NEAVE
875 THIRD AVENUE 29TH FLOOR
NEW YORK, NY 10022

EXAMINER	
CHAN, N	
ART UNIT	PAPER NUMBER
123	[24]*

DATE MAILED: 04/16/87

This is a communication from the examiner in charge of your application.
COMMISSIONER OF PATENTS AND TRADEMARKS

☐ This application has been examined

☒ Responsive to communication filed on [1/5/87]

☒ This action is made final.

A shortened statutory period for response to this action is set to expire [3] month(s), _____ days from the date of this letter. Failure to respond within the period for response will cause the application to become abandoned. 35 U.S.C. 133

---

* All text in brackets was handwritten in the original.

Part I THE FOLLOWING ATTACHMENT(S) ARE PART OF THIS ACTION:

1. ☐ Notice of References Cited by Examiner, PTO-892
2. ☐ Notice re Patent Drawing, PTO-948
3. ☐ Notice of Art Cited by Applicant, PTO-1449
4. ☐ Notice of informal Patent Application, Form PTO-152
5. ☐ Information on How to Effect Drawing Changes, PTO-1474
6. ☐ _____

Part II SUMMARY OF ACTION

1. ☒ Claims [145, 148-150, 155, 162-164, 166, 171-174, 177, 179-181, 183-184, 189-192, and 199] are pending in the application.

   Of the above claims _____ are withdrawn from consideration.
2. ☐ Claims _____ have been cancelled.
3. ☐ Claims _____ are allowed.
4. ☒ Claims [145, 148-150, 155, 162-164, 166, 171-174, 177, 179-181, 183-184, 189-192 and 199] are rejected.

* * * * *

Serial Number 496915

Art Unit 123

Claims 145, 148-150, 155 162-164, 166, 171-174, 177, 179-181, 183, 184, 189-192 and 199 are in the case.

Applicant's arguments filed January 5. 1987 have been fully considered but they are not deemed to be persuasive.

Claims 145, 148-150, 155, 162-164, 166, 171-174, 177, 179-181, 183-184, 189-192 and 199, all of the claims in case, are rejected under 35 U.S.C. 102 (a) as anticipated by or, in the alternative, under 35 U.S.C. 103 as obvious over [La]nger et al abstract 1153.

This symposium abstract evidences that the claimed invention [was] known by other in this country before the invention thereof by the applicant.

A declaration under 37 CFR 1.131 as offered by applicant will obviate this rejection.

Claims 177, 179-180 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

In claim 177, the word "deazaadenine" is misspelled. [not in cl 17. Told Oster to forget it. 6/1/87]

In claim 179, the recitation of A as "as second moiety" is confusing because A is included in the "first moiety". The word "second" shoudl be deleted.

A bond connecting the OH group is missing.

The specification should be amended to reflect the status of the parent application and should be reviewed for any minor errors.

Serial Number 496915

Art Unit 123

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a). The practice of automatically extending the shortened statutory period an additional month upon the filing of a timely first response to a final rejection has been discontinued by the Office. See 1021 TMOG 35.

A SHORTENED STATUTORY PERIOD FOR RESPONSE TO THIS FINAL ACTION IS SET TO EXPIRE THREE MONTHS FROM THE DATE OF THIS ACTION. IN THE EVENT A FIRST RESPONSE IS FILED WITHIN TWO MONTHS OF THE MAILING DATE OF THIS FINAL ACTION AND THE ADVISORY ACTION IS NOT MAILED UNTIL AFTER THE END OF THE THREE-MONTH SHORTENED STATUTORY PERIOD, THEN THE SHORTENED STATUTORY PERIOD WILL EXPIRE ON THE DATE THE ADVISORY ACTION IS MAILED, AND ANY EXTENSION FEE PURSUANT TO 37 CFR 1.136(a) WILL BE CALCULATED FROM THE MAILING DATE OF THE ADVISORY ACTION. IN NO EVENT WILL THE STATUTORY PERIOD FOR RESPONSE EXPIRE LATER THAN SIX MONTHS FROM THE DATE OF THIS FINAL ACTION.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Dr. Nicky Chan whose telephone number is (703) 557-0367.

Any inquiry of a general nature or relating to the status of this application should be directed to the Group receptionist whose telephone number is (703) 557-3920.

NChan:cc
4-14-87

/s/
Nicky Chan
Primary Examiner
Art Unit 123

APPENDIX F

LETTER OF MARCH 7, 1995 FROM DR. STEPHEN MACEVICZ TO HON. LAWRENCE J. GOFFNEY, ASSISTANT COMMISSIONER OF PATENTS

PERKIN ELMER

Applied Biosystems Division
850 Lincoln Centre Drive
Foster City, CA 94404

7 March 1995

VIA FACSIMILE **7 PAGES IN TRANSMISSION**

Hon. Lawrence J. Goffney, Jr.
Assistant Commissioner of Patents
U.S. Department of Commerce
Washington, D.C. 20231

Subject: **SUBMARINE PATENT APPLICATION, a continuation of Ward et al, USSN 06/496,915 filed 23 May 1983 (Ward I) and/or Ward et al, USSN 07/130,070 filed 8 December 1987 (Ward II) (Cover sheets attached)**

Dear Sir:

It was a pleasure meeting with you and several of my colleagues from Silicon Valley last Friday, March 3. We were very encouraged by your remarks and your commitment to improving Patent Office performance. I personally very much appreciated the opportunity to tell you

about the particular difficulty that my company is facing because of a Patent Office "quality control" issue.

In this communication, I want to re-iterate my concern and again ask if something could be done to address this concern *inside the Patent Office* prior to the issuance of a third Ward patent. I greatly fear that a mis-application of patentability standards is leading my company into a grossly unfair multi-million dollar patent infringement suit. My concern about the Ward application is summarized below:

SOURCE OF CONCERN:

Letter from Enzo Biochem stating that the U.S. Patent Office issued a Notice of Allowance for Claim 195 attached as Exhibit A.

This claim covers fluorescently labeled DNA sequencing fragments (formula in terms of Claim 195 shown on Exhibit B), a key component in all presently and foreseeably available automated DNA sequencing procedures and instruments--that is, a fundamental technology upon which all genome sequencing projects rely.

This is a $200M+ per year business, largely built by Applied Biosystems. Many small-to-medium-sized biotech instrumentation companies, besides Applied Biosystems, would be severely damaged by the issuance of Claim 195, or like claims.

WHAT IS THE PATENTABILITY ISSUE:

Claim 195 combines disparate elements of the original disclosure (and invention) to create a new compound not described in the specification as required under 35 U.S.C. 112 first paragraph. **This newly described compound inappropriately covers DNA sequencing fragments.** No prior claim of the application or passage in the

specification describes compounds that cover DNA sequencing fragments.

The most compelling facts of this situation are illustrated by the time line of Exhibit C: The original Ward application was filed nearly four years prior to the invention of fluorescent DNA sequencing. Nothing in any of the Ward specifications, the prosecution up to 1994, or pending claims up to 1994 give any indication that the invention includes compounds covering DNA sequencing fragments. It is only after the industry grew from $0 in sales to over $200 million in sales between 1986 and 1995 that a claim appeared in a recent continuation application that sought to cover DNA sequencing fragments! These facts are characteristic of the so-called SUBMARINE PATENT phenomena.

* * * * *

I hope you find the above information and explanations helpful. Our industry would greatly appreciate any help you could provide in rectifying this unfortunate situation.

With best regards,

/s/

Stephen C. Macevicz
Senior Patent Attorney

Attachments
cc: Dr. Michael Hunkapiller
Vice President